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JC930 U.S. PTO 09/717450 11/20/00

Docket No. 0630/0D532US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lisa Ann NEUHOLD and Loren KILLAR

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASE OF CARTILAGE

09717450 .112000

CONTINUING APPLICATION

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

This is a request for filing under 37 C.F.R. 1.53(b) of a:

[X] Continuation [] Divisional

application of the following pending prior application:

Serial No. 08/994,689 Filed: 12/19/97

Of: NEUHOLD et al.

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASE OF CARTILAGE

Examiner: M. Wilson Group: 1633

1. Enclosed is a copy of the prior application as originally filed (along with a copy of the original Declaration). No amendments identified in the

declaration for this prior application introduced new matter. Please use this copy as the application and declaration for the present case.

2. The filing fee is calculated below:

CLAIMS AS FILED, AFTER ACCOMPANYING AMENDMENT

	Claims on File	Number Extra	Rate
Basic Fee			$710.00
Total Claims	27- 20 =	x $	$126.00
Independent Claims	2- 3 =	x $	$
If Multiple Dependent Claims Are Present, Add $.00			$
Total Filing Fee			$836.00
For Small Entity (half of preceding total)*			$

*No. 9 below must be checked to claim this reduction.

3. A check in the amount of $836.00 is enclosed.

4. [] Cancel claims _.

5. [X] Amend the specification by inserting before the first line the sentence (check one and fill in):

"This is a [X] continuation, [] division, of application Serial No. 08/994,689, filed 12/19/97. Each prior application is hereby incorporated herein by reference, in its entirety."

6. [X] The prior application is assigned to: American Home Products Corporation

7. [X] A Preliminary Amendment is also enclosed.

Docket No. 0630/0D532

8. [X] Informal Drawings are filed herewith

9. [] A verified statement claiming small entity status (check one):
[] was filed in parent application or [] is enclosed.

10. [] Priority is claimed from
Country:
Number:
Date:

The priority document
[] was filed in the prior application
[] is enclosed.

11. The Assignment
[X] was recorded at Reel 8934 Frame 0165 on December 19, 1997
[] is enclosed.

12. Sequence Listing

Pursuant to 37 C.F.R. § 12.82(e), please use the computer readable form (CRF) of the "Sequence Lising" from parent application Serial No. 08/944,689, filed December 19, 1997, in this application. The paper copy of the "Sequence Listing" in this application is identical to CRF filed for the parent application.

Respectfully submitted,

Paul F. Fehlner, Ph.D.
Reg. No. 35,135
Attorney of Record in Prior Application

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M:\0630\1D532US1\CB4521 WPD

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D B Peck
Name (Print) Signature

Docket No: **0630/1D532US1**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lisa Ann NEUHOLD and Loren KILLAR

Serial No.: To Be Assigned, Continuation of Serial No. 08/944,689

Filed: Concurrently Herewith

For: **TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE**

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

Please consider the following amendments and remarks with the continuation application.

IN THE CLAIMS:

Please cancel claims 1-27 without prejudice.

Please add the following claims:

--28. A transgenic non-human mammal or progeny thereof whose somatic and germline cells contain, in stably integrated form,

(a) a first coding sequence encoding an enzymatically active matrix degrading enzyme (MDE) that degrades an extracellular matrix component, wherein expression of the first coding sequence is under control of a regulatable promoter that is responsive to a transcriptional repressor or activator polypeptide; and

(b) a second coding sequence encoding the transcriptional repressor or activator polypeptide, wherein expression of the second coding sequence is under control of a chondrocyte tissue-specific promoter;

wherein expression of the MDE by chondrocytes is repressed throughout embryonic, fetal, and early postnatal development, and activation of expression of the MDE results in a phenotypic change characteristic of osteoarthritis.

29. The transgenic mammal of claim 28, wherein the MDE is a matrix metalloproteinase selected from the group consisting of MMP-1, MMP-3, MMP-8, and MMP-13.

30. The transgenic mammal of claim 28, wherein the MDE is constitutively enzymatically active without proteolytic processing.

31. The transgenic mammal of claim 30, wherein the MDE is a constitutively enzymatically active MMP-13 variant.

32. The transgenic mammal of claim 31, wherein the MMP-13 variant has a sequence of ID NO:1 or SEQ ID NO:21.

33. The transgenic mammal of claim 28, wherein the mammal is selected from the group consisting of a mouse, a rat, and a rabbit.

34. The transgenic mammal of claim 28, wherein the mammal is a mouse.

35. The transgenic mammal of claim 28, wherein the transcriptional repressor or activator polypeptide is a repressor polypeptide.

36. The transgenic mammal of claim 35, wherein the repressor polypeptide is a tetracycline repressor polypeptide.

37. The transgenic mammal of claim 36, wherein the regulatable promoter comprises a tet07 sequence.

38. The transgenic mammal of claim 37, wherein the regulatable promoter comprises a sequence depicted in SEQ ID NO:2.

39. The transgenic mammal of claim 28, wherein the chondrocyte tissue-specific promoter comprises sequences from a Type II collagen promoter.

40. The transgenic mammal of claim 28, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

41. A transgenic mouse or rat, or progeny thereof, whose somatic and germline cells contain, in stably integrated form,

(a) a first coding sequence encoding a constitutively enzymatically active matrix metalloproteinase (MMP) that cleaves Type II collagen, wherein expression of the first coding sequence is under control of a tetracycline-regulatable promoter; and

(b) a second coding sequence encoding a tetracycline repressor polypeptide that binds to the tetracycline-regulatable promoter, wherein expression of the second coding sequence is under control of a chondrocyte tissue-specific promoter;

wherein expression of the MMP by chondrocytes is repressed throughout embryonic, fetal, and early postnatal development, and activation of expression of the MMP results in a phenotypic change characteristic of osteoarthritis in the transgenic mouse or rat.

42. The transgenic mouse or rat of claim 41, wherein the MMP is constitutively enzymatically active MMP-13, the tetracycline regulatable promoter is a tet07 promoter, the tetracycline repressor polypeptide is a tTA polypeptide, and the chondrocyte tissue-specific promoter comprises sequences from a Type II collagen promoter.

43. The transgenic mouse or rat of claim 42, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

44. A method for producing a phenotypic change characteristic of osteoarthritis in a transgenic mammal of claim 28, which method comprises activating MDE expression in the transgenic mammal after embryonic, fetal, and early postnatal development.

45. The method according to claim 44, wherein the phenotypic change

characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

46. A method for producing a phenotypic change characteristic of osteoarthritis in the transgenic mammal of claim 36, which method comprises maintaining the transgenic mammal on tetracycline or a tetracycline analog during embryonic, fetal, and early postnatal development, and activating the MDE expression by withholding the tetracycline or tetracycline analog after embryonic, fetal, and early postnatal development.

47. The method according to claim 46, wherein the tetracycline analog is doxycycline.

48. The method according to claim 46, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

49. A method for producing a phenotypic change characteristic of osteoarthritis in the transgenic mouse or rat of claim 41, which method comprises maintaining the transgenic mouse or rat on tetracycline or a tetracycline analog during embryonic, fetal, and early postnatal development, and activating the collagenase expression by withholding the tetracycline or tetracycline analog after embryonic, fetal, and early postnatal development.

50. The method according to claim 49, wherein the tetracycline analog is doxycycline.

51. The method according to claim 49, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

52. A method for evaluating potential of a composition to counteract a phenotypic change characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mammal of claim 28 in which a phenotypic change characteristic of osteoarthritis has been produced by activation of expression of the MDE after embryonic, fetal, and early postnatal development of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered relative to a control mammal in which expression of the MDE was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.

53. A method for evaluating potential of a composition to counteract a phenotypic characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mammal of claim 36 in which a phenotypic change characteristic of osteoarthritis has been produced by activating expression of the MDE by withholding tetracycline or a tetracycline analog after embryonic, fetal, and early postnatal development of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered relative to a control mammal in which expression of the MDE was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.

54. A method for evaluating potential of a composition to counteract a phenotypic change characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mouse or rat of claim 41 in which a phenotypic change characteristic of osteoarthritis has been produced by activating expression of the MMP by withholding tetracycline or a tetracycline analog after embryonic, fetal, and early postnatal development of the transgenic mouse;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mouse or rat to which the composition was administered relative to a control mouse or rat in which expression of the MMP was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.--

REMARKS

This Application is a Continuation of Serial No. 08/994,689 (the '689 application), which has been finally rejected in an Office Action dated September 29, 2000. The claims in this preliminary rejection are substantively identical to the claims finally rejected in the '689 application. Some changes to address clarity rejections have been made to the claims.

The claims in the application as filed have been canceled and new claims 28-54 have been added to more particularly point out and distinctly claim that which applicants regard as the invention. The new claims are fully supported by the claims as filed and by the specification. In particular, support for new claims 29-55 is set forth in the following table:

New Claims	Support
28.	Claim 1 as filed; p. 27, l. 9 (coding sequence); at p. 2, l. 4 and p. 5, ll. 19-20 (enzymatically active matrix degrading enzyme (MDE) that degrades an extracellular matrix component); p. 6, ll. 11-12 (regulatable promoter); p. 13, ll. 6-8 (promoter that is responsive to a transcriptional repressor or activator polypeptide); p. 37, l. 1, p. 6, ll. 4-5, p. 13, ll. 3-4 (chondrocyte tissue-specific promoter); p. 13, l. 1, p. 16, ll. 12-15 (expression repressed throughout embryonic, fetal, and early postnatal development); p. 6., l. 5-6, p. 18, ll. 9-11 (MDE expression results in a phenotypic change characteristic of osteoarthritis).
29.	p. 2, Table 1 and p. 5, ll. 23-24.
30.	p. 12, l. 8.
31.	p. 12, l. 13.
32.	claim 7 as filed; p. 12, ll. 16-18.
33.	claim 8 as filed.
34.	claim 9 as filed.

35.	p. 13, l. 10.
36.	p. 14, l. 21.
37.	claim 11 as filed; p. 14, ll. 21-22
38.	claim 12 as filed
39.	claim 15 as filed; p. 16, l. 2.
40. (also 43, 45, 48, and 51)	p. 18, ll. 13-15; p. 19, ll. 11-18; p. 21, ll. 5-6.
41.	p. 27, l. 9 (coding sequence); p. 12, ll. 8-12 (constitutively active MMP); p.3, ll. 4-5, p. 11, ll. 5-6 (degrades Type II collagen) p. 6, ll. 11-12; p. 13, ll. 6-8 and 19; (tetracycline regulatable promoter); p. 13, l. 19 (tetracycline repressor polypeptide); p. 37, l. 1; p. 6, ll. 4-5; p. 13, ll. 3-4 (chondrocyte tissue-specific promoter); p. 13, l. 1 and p. 16, ll. 12-15 (expression repressed throughout embryonic, fetal, and early postnatal development); p. 18, ll. 9-11 (MMP expression results in phenotypic change characteristic of osteoarthritis).
42.	claim 16 as filed; p. 17, l. 24 to p. 18, l. 2.
44. (also 46 and 49)	claims 22-24 as filed; p. 18, ll. 16-21.
47. (also 50)	p. 41, l. 6.

52-54	claims 25-27 as filed; p. 20, l. 13 to p. 21, l. 2.

SUMMARY OF POINTS

Based on the grounds for rejection in the outstanding Final Office Action in the '689 parent application, and the discussion at the interview in the '689 parent application conducted on May 17, 2000, applicants submit that there are no issues with respect to patentability of the specific transgenic mice exemplified in this application. However, as discussed at the interview, such a limited claim scope denies the protection to which this discovery is entitled: any transgenic animal that expresses an enzymatically active matrix degrading enzyme under control of an inducible promoter and a tissue-specific promoter, and, when induced to express the enzymatically active matrix degrading enzyme, develops phenotypic changes characteristic of osteoarthritis. Applicants wish to emphasize the following points in support of patentability of the invention as presently claimed:[1]

- Enzymatically active matrix degrading enzymes that degrade extracellular matrix components are well known. Applicants have established this in the file history of the application as follows:

  Specification: pages 2-3; pages 11-12.

  Additional evidence: Second Neuhold Declaration, paragraph 8.

- Expression of coding sequences under control of regulatable promoters that are responsive to a transcriptional repressor or activator polypeptide in transgenic

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[1] Applicants refer herein to the Declaration of Lisa A. Neuhold, Ph.D. Under 37 C.F.R. § 1.132 filed April 6, 1999 (the Neuhold Declaration; copy attached as Exhibit A), the preliminary Amendment filed by hand on February 18, 2000 (the Preliminary Amendment; copy attached as Exhibit B) and the Second Declaration of Lisa A. Neuhold, Ph.D. Under 37 C.F.R. § 1.132 filed August 30, 2000 (the Second Neuhold Declaration; copy attached as Exhibit C), filed in the '689 parent application.

animals is well known. Applicants have established this in the file history of this application as follows:

Specification: pages 12-15 and pages 16-18.
Additional Evidence: Exhibits A, B, C, D, and E to Preliminary Amendment; Second Neuhold Declaration, paragraph 6.

- Tissue specific expression of transgenes in transgenic animals is well known. Applicants have established this in the file history of this application as follows:

  Specification: pages 15-16 and 17-18.
  Additional Evidence: Neuhold Declaration, paragraph 6; Second Neuhold Declaration, paragraph 7 and Tab 2.

- Transgenic nonhuman mammals, particularly mice, rats, and rabbits, are well known and prepared routinely by ordinary research scientists at the time this invention was made. It is also a known and accepted that, as with every other experimental system in biology such as cloning and hydridomas, not every transgenic embryo will yield a transgenic animal with the desired characteristics, but that routine screening and selection techniques will provide such an animal as claimed. Applicants have established this extensively in the file history of this application as follows:

  Specification: pages 22-26.
  Additional Evidence: Second Neuhold Declaration, paragraph 9 and Tab 4.

- Phenotypic characteristics of osteoarthritis (degenerative bone disease), as developed in the transgenic animals of the invention are well-known. Applicants have established this in the file history of the application as follows:

  Specification: pages 18-20, 21 and 45.
  Additional Evidence: Neuhold Declaration,
  paragraphs 6, 11, 13 and 14; Exhibits F, G, H, and I
  to the Preliminary Amendment; Second Neuhold
  Declaration, paragraph 10.

- The totality of the evidence of record in the file history of this application establishes that the claimed transgenic mammals, particularly the claimed transgenic mice and rats, can be generated and induced to develop one or more phenotypic characteristics of osteoarthritis. These transgenic animals thus serve as useful models for studying the progression and evaluating therapies for this disease. Applicants have established these features of the invention throughout the file history of the application as set forth and as follows:

  Specification: pages 1, 5-7, 18-20, 21 and 45.
  Additional Evidence: Neuhold Declaration,
  paragraph13; Second Neuhold Declaration,
  paragraph 11.

<u>THE SPECIFICATION ENABLES THE CLAIMED INVENTION</u>

In the '689 application, the Examiner had rejected claims 29-55 under 35 U.S.C. § 112, first paragraph, contending that while being enabling only for MMP13* (SEQ ID NO:1) linked to any regulatable promoter, *e.g.*, tet07 promoter + tet repressor and VP16 activator,

linked to Type II collagen promoter where a mouse is given the regulatory compound, *e.g.*, tetracycline until adulthood, the specification does not provide enablement for a mammal.

Applicants respectfully traverse this rejection. For the reasons advanced above in the accompanying Second Neuhold Declaration, the specification enables claims to mammals. In particular, ". . . contrary to the examiner's assertions, as of 1996 creation of transgenic mammals required no more than ordinary technical efforts – indeed, technical efforts with shortcomings that are readily overcome" (Neuhold Declaration, paragraph 9).[2] All of these techniques are set forth in the specification at pages 22-26.

Notably, the present invention discloses, *inter alia*, microinjection of zygotes, viral integration, and transformation of embryonic stem cells "as methods for introducing transgenes into animals (specification, page 23, lines 11-12). "Microinjection of zygotes in the preferred method" (page 23, line 13; see, page 23, line 13 to page 24, line 3), which the specification exemplifies (see page 38, lines 13-19)[3] for the reasons discussed in greater detail below, the state of the art at the time this invention was made was much farther advanced than the Examiner allows, and the Examiner's contention is incorrect.

The Examiner further contended that the specification does not teach how to get phenotype with any other regulating system. As discussed during the interview and set forth in the accompanying Second Neuhold Declaration at paragraph 6, the regulatable expression systems described in the specification are well established and well known in the art. As set forth above, applicants have submitted numerous references further supporting enablement of this aspect of the invention. The Examiner appeared to agree with this position at the interview and in the Final Office Action. Accordingly, this basis for the rejection is mooted.

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[2] The ‘689 Final Office Action does not address this fact.

[3] The Examiner's focus on ES-cell approaches to creation of transgenic animals seems particularly misplaced in view of these facts.

The Examiner also contended that the specification does not teach how to get other phenotypes. Applicants disagree; the record as set forth above and the Second Neuhold Declaration (paragraphs 10 and 11) firmly establishes that the transgenic mammals, and especially rodents, of the invention, upon expression of the matrix degrading enzyme, develops a phenotypic change characteristic of osteoarthritis.

The Examiner contended that it is not clear whether other extracellular matrix degrading enzymes would achieve the claimed phenotype. Applicants respectfully disagree. The specification sets forth a plethora of matrix degrading enzymes (pages 2-3); the Examiner has provided no evidence or documentation to substantiate doubts that other MDEs would achieve this phenotype. It is uncontroverted that other Type II collagenase enzymes, such as MMP-1, MMP-8, and MMP-13, would (see claim 41). The Examiner has the burden to establish a <u>reasonable</u> basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). MPEP § 2164.04. The Examiner clearly failed to meet this burden here.[4] In contrast, Applicants have more than met theirs: in addition to the express disclosure of the specification (see pages 2-3 and 11-12), they have further addressed this issue in the interview and by Rule 132 Declaration (*see* the Second Neuhold Declaration, paragraph 8). Thus, this rejection is overcome and should be withdrawn. Claim 41 particularly addresses these concerns by reciting a constitutively active collagen II-specific MMP.

The Examiner asserted that the invention is unpredictable with regard to phenotype. However, the evidence of record in the specification (pages 41-44), the Neuhold Declaration (paragraphs 9, 11, 13, 14, and 15), and the Second Neuhold Declaration (paragraphs 10 and 11) firmly establish that the transgenic animals of the invention demonstrate the claimed phenotypic change, *i.e.*, one or more characteristics of osteoarthritis.

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4 The '689 Final Office Action does not address this defect.

The Examiner also asserted that non-mutated MMP genes are not enabled. As these proteins, and methods for activating them, are extremely well known in the art (as agreed at the interview), which is specifically established on pages 2-3 of the specification, Applicants submit that this rejection is obviated and should be withdrawn. Furthermore, this rejection does not properly apply to claim 41.

The Examiner further contended that the specification is only enabled for MMP13* and exemplified phenotype. For the reasons set forth above, Applicants submit that the specification broadly enables transgenic mammals that express any MDE in a tissue specific, temporally regulated fashion. Furthermore, as the exemplified phenotype is a phenotypic change characteristic of osteoarthritis, which includes cartilage degradation (Second Neuhold Declaration, paragraphs 10 and 11), the specification clearly enables this aspect of the claimed invention as well. Thus, these bases for rejecting the claims are obviated and should be withdrawn.

The Examiner contended that the specification is not enabled for any joint specific promoter and should recite only a Type II collagen promoter. As discussed during the interview and set forth in the Second Neuhold Declaration (paragraph 7), the specific promoter employed to achieve tissue specific expression does not make any difference, as one of ordinary skill in the art would readily appreciate. A number of issued patents that cover transgenic animals establish tissue-specific expression is sufficiently enabled for expression of a transgene, because the actual tissue specific promoter is usually of no moment. Moreover, it is proper in a patent for a transgenic animal to claim the promoter by virtue of its tissue specificity rather than identity. *See* U.S. Patent Nos. 5,625,124 (claim 1: "gut epithelial cell specific promoter"); 5,880,327 (claim 1: "a mammary-gland specific promoter"); 5,917,123 (claim 1: "a cardiac-specific regulatory region"); 6,028,245 (claim 1: "a promoter that drives expression of the transgene in skin")

(attached as Exhibit 2). In view of the foregoing, the Examiner's basis for this rejection is obviated and should be withdrawn.[5]

The Examiner cited references detailing expression in other mammals as support for argument that the specification does not enable non-human mammals besides mouse. Applicants respectfully take issue with these citations on two grounds. First, they support the opposite conclusion: that the claimed transgenic animals are enabled. Second, these references do not adequately support the rejection. Applicants submit that the Examiner has not carried this burden where the support (1) generally refers to the generic technology; (2) addresses questions related to economics and commercialization, not § 112, first paragraph; (3) contains no information specifically relevant to the claimed invention; and (4) is out of date (none of the references cited by the Examiner have publication dates later than 1996; one was published in 1988; in a rapidly evolving field such as transgenic animals, only the most current references from the time the invention was made have any bearing). *Cf. In re Goodman*, 29 USPQ2d 2010 (Fed. Cir. 1993) (applying references directly addressing the claims in issue to establish lack of enablement).[6]

In particular, the Wall reference reports that 6000 papers describe transgenic animals, mostly mice, to answer research questions (pages 58, 60, and 61). Wall states that ". . . genes can . . . be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered)" (page 58). In other words, Wall specifically states that the features of Applicants' invention can be achieved. Wall does concede that transgenic farm animals are costly (mostly because it takes many attempts to yield the desired transgenic animal) (see page 60), however, economic issues are irrelevant to

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5 The '689 Final Office Action fails to address any of these issues.

6 The '689 Final Office Action does not address any of these issues, or Applicants' contention that the references clearly support enablement, as discussed further.

enablement. How is it possible that a reference acknowledging such an abundance of research papers on transgenic animals, manipulation of expression, and at least 1% efficiency of obtaining the desired transgenic animal (much higher, one might add, than the likelihood of obtaining a desired monoclonal antibody or even cloning a gene) calls into question enablement of this invention? On the contrary, Applicants might very well (and here do) cite such a reference to support the routine nature of generating experimental transgenic animals for disease models.

The Ebert reference (from 1988) reports success ("Transgenic pigs carrying this fusion gene had elevated levels of circulating human somatrotropin"; page 277). Applicants submit that the presence of failures is irrelevant in the face of success. The entire history of biology is one of selecting and screening for successes from the much more abundant failures. *See In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988) (enablement of broad monoclonal antibody claims despite the large number of trials necessary to obtain the operative antibody).

Mullins and Mullins, like Wall, report that transgenic technology, including ES technology, is well established (page S37). Time and cost, issues irrelevant to enablement, limit the desirability of pronuclear injection in larger mammals. No matter, as pointed out in the specification, ES technology is an alternative. In any event, the fact that pronuclear injection is less efficient, and therefore economically undesirable, fails to establish that it does not work. On the contrary, nothing in Mullins and Mullins supports such a conclusion. In any event, this paper reports on a number of successful non-murine transgenic animal models (see page S38).

Finally, the Overbeck reference shows that different transgenic animals will demonstrate different levels of expression. Regulatory sequences help avoid variability (see page 97), but this makes little difference: variability ranges from one extreme to another, from no phenotypic change to the desired change. The Examiner contends that this establishes unpredictability. Applicants disagree. This establishes predictability of two things: there will be failures, and there will also be successes. By selecting the successes, which is routine, one

achieves the desired transgenic animals. Indeed, applicants themselves had failures, among which successful animals were obtained (see page 43 of the specification).

In short, the Examiner's grounds for rejection are in error given the advanced state of the art, including general recognition of enablement of transgenic animals (irrespective of whether or not they are cost effective), widespread knowledge of regulatable expression systems, the understanding in the art of tissue-specific expression, and the number of well known extracellular matrix degrading enzymes from which to choose. The present invention is broadly enabled, and the Examiner has not met his burden of challenging enablement with reasonable evidence. Accordingly, the rejection under 35 U.S.C. § 112, first paragraph is in error and should be withdrawn.

CONCLUSION

Because the claims of this application, which is a continuing application of the '689 application, are drawn to the same invention claimed in the earlier application and would have been properly [sic, procedurally][7] finally rejection on the grounds of record in the next Office Action if they had been entered in the '689 application. Accordingly, a Final Rejection is appropriate as a First Office Action in this Continuation application. MPEP § 706.07(b) (Rev. 1, Feb. 2000). Accordingly, if the Examiner persists in rejecting these claims, applicants believe a Final Rejection is appropriate and necessary to permit appeal.

Applicants respectfully request entry of the foregoing amendments and remarks as well as the amendments and Rule 132 Declarations from the '689 application in the file history of this application. In view of the foregoing amendments and remarks, applicants submit that the

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[7] Applicants use the term properly in a procedural context, and do not agree with or concede the rejections in '689.

claims meet all the statutory requirements for patentability. If the Examiner has any other concerns, he is invited to contact the undersigned by telephone. Allowance or Final Rejection of the claims is earnestly solicited.

Respectfully submitted,

Date: November 20, 2000

Paul F. Fehlner, Ph.D.
Reg. No.: 35, 135
Attorney for Applicants

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805 Third Avenue
New York, N.Y. 10022
Phone (212) 527-7700

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0630/0D532

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Date 12-1997 Label No. EM 595861576

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G KARASZI — Name (Print)

Signature

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D B Peek — Name (Print)

Signature

TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

Field of the Invention

The present invention pertains to transgenic mammals that express recombinant matrix-degrading enzymes in a temporally and spatially regulated manner. The invention further pertains to model systems incorporating such transgenic mammals for studying degenerative joint diseases, including systems for identifying therapeutic agents and treatment regimens.

Background of the Invention

Degenerative diseases of cartilage, including joint and disc diseases such as osteoarthritis, rheumatoid arthritis, and osteochondrodysplasias, are widespread, particularly in the elderly. Early symptoms common to these diseases include progressive loss of proteoglycans in the joint (as evidenced by loss of metachromasia); collagen degradation; fibrillation of the cartilage surface; and, ultimately, loss of cartilage (which is evidenced radiologically as joint space narrowing).

One of the primary targets affected by these diseases is type II collagen, the major structural collagen found in articular cartilage. There is a balance between the

production of type II collagen and catabolic enzymes that degrade type II collagen during normal remodeling of cartilage and bone. Pathological conditions such as, e.g., degenerative joint diseases, may result when this balance is disrupted.

Among the enzymes that degrade extracellular matrix components are matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, and aggrecanase (Table 1).

Table 1

Matrix-Degrading Enzymes							
	SUBSTRATES						
	Collagen	*Gelatin*	*Proteoglycan*	*Fibronectin*	*Laminin*	*Elastin*	*Other*
I. Metalloproteinases							
Collagenases							
MMP-1 (intestinal collagenase)	I, II, III, VII, X	✓					
MMP-8 (neutrophil collagenase)	I, II, III						
MMP-13 (collagenase 3)	I, II, III	✓					
Gelatinases							
MMP-2 (gelatinase A)	IV, V, VII, XI	✓		✓	✓	✓	
MMP-9 (gelatinase B)	IV, V	✓	✓				
Stromelysins							
MMP-3 (stromelysin 1)		✓	✓	✓	✓		activates MMP zymogens
MMP-7 (matrilysin)	IV	✓	✓	✓	✓	✓	
MMP-10 (stromelysin 2)	IV, V, IX		✓	✓	✓		activates MMP zymogens
MMP-11 (stromelysin 3)	IV			✓	✓		activates serpins

Matrix-Degrading Enzymes							
	SUBSTRATES						
	Collagen	*Gelatin*	*Proteoglycan*	*Fibronectin*	*Laminin*	*Elastin*	*Other*
Other							
MMP-12 (metalloelastase)						✓	
MMP-14		✓					proMMP-2, proMMP-13
MMP-15							
MMP-16							proMMP-2
MMP-17							
II. Aggrecanase			✓				

MMPs are synthesized in articulating joints by chondrocytes, which, in mature articular cartilage, are terminally differentiated cells that maintain the cartilage-specific matrix phenotype. Overexpression of MMPs relative to endogenous MMP inhibitors, as occurs in degenerative joint diseases, may result in cartilage degradation. For example, Type II collagen is a substrate for MMP-13 and MMP-1 (Knauper et al., *J. Biol. Chem.* 271:1544, 1996) and both MMP-1 and MMP-13 proteins can be detected immunohistochemically in human osteoarthritic tissues. In some cases, MMP-13 and its cleavage products are found at higher levels than MMP-1. Billinghurst et al., *J. Clin. Inves.* 99:1534, 1997. Thus, MMP-13 may play an important role in cartilage degradation associated with osteoarthritis and other degenerative joint diseases. (Mitchell et al., *J. Clin. Inves.* 97:761, 1996).

Animal models for osteoarthritis-related syndromes have been described in guinea pigs (Watson et al., *Arth. Rheum.* 39:1327, 1996) and in the inbred STR/ORT strain

of mice (Das-Gupta et al., *Int. J. Exp. Path.* 74:627, 1993). In guinea pigs, spontaneous osteoarthritis has a long course of development (six months or more), and only certain sublines of STR/ORT mice consistently develop degenerative joint disease. Thus, the duration and/or variability of these models renders them less applicable to drug discovery studies.

Other osteoarthritis-related models include surgically-induced joint destabilization, e.g., anterior cruciate ligament transection and/or partial meniscectomy in rabbits and dogs, which stimulates cartilage degradation. Hulth et al., *Acta Orthop. Scand.* 41:522, 1970. Another model employs injection of bacterial collagenase into the joints of an animal to induce a biochemical ligament transection. Van der Kraan et al., *J. Exp. Pathol.* 71:19, 1990. Because (i) surgical or other manipulation of individual animals is required; (ii) the animals are large and expensive; and/or (iii) the course of disease is not consistent, these models cannot easily be used in large-scale studies, including drug screening.

Transgenic animal models, in principle, can provide the opportunity for a reproducible animal model system for degenerative joint diseases. However, previous attempts to engineer transgenic animals expressing MMPs such as MMP-1 and stromelysin have not resulted in an observable joint degeneration phenotype in the transgenic animals. This could be due to embryonic lethality caused by constitutive expression of these enzymes. Witty et al., *Mol.Biol. Cell* 6:1287, 1995, have created transgenic animals that constitutively express MMP-1 and stromelysin in mammary tissue, but these animals do not exhibit symptoms of osteoarthritis. D'Armiento et al., *Cell* 71:955, 1992, disclose transgenic mice that express human interstitial collagenase in the lung. Liu et al., *J. Cell Biol.* 130:227, 1995, disclose transgenic animals that overexpress mutated type II collagen, resulting in

connective tissue defects but not osteoarthritis. None of these transgenic animal systems provides a useful animal model for osteoarthritis. Khokha et al., *Cancer and Metastasis Rev.* 14:97, 1995; Shapiro, *Matrix Biol.* 15:527, 1997.

Thus, there is a need in the art for animal model systems that mimic human degenerative joint diseases such as, e.g., osteoarthritis, rheumatoid arthritis, and chondrodysplasias. Transgenic animals containing regulatable heterologous genes whose expression results in cartilage degeneration are particularly advantageous in providing reproducible experimental control over the timing and the level of expression of the transgenes and, thereby, over the pathological syndrome itself. Such animals can be used to determine what level of expression of the transgene is required to cause disease and, importantly, can be used for drug discovery and optimization of treatment regimens. In particular, such transgenic animals can be used to further define the role of matrix-degrading enzymes in cartilage degradation and as an *in vivo* screen to identify compounds that modulate these enzymes or compounds that inhibit the progression of degenerative joint diseases.

Summary of the Invention

The present invention provides transgenic non-human animals or the progeny thereof whose somatic and germline cells contain, in stably integrated form, one or more heterologous or recombinant genes encoding polypeptides comprising enzymatically active matrix-degrading enzymes (MDEs), preferably MMPs. MMPs for use in the invention comprise one or more of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16, and MMP-17; preferably one or more of MMP-1, MMP-3, MMP-8, and MMP-13; and most preferably one or more of

MMP-1 and MMP-13; and include enzymatically active variants, fragments, and combinations of these polypeptides. Other matrix-degrading enzymes can also be used, including, e.g., aggrecanase. The MDEs may be derived from any species, preferably human. In preferred embodiments, the recombinant MDE-encoding genes are selectively expressed in articular chondrocytes of the transgenic animal and expression results in pathological symptoms characteristic of degenerative joint disease.

In one aspect, the invention provides a transgenic animal or the progeny thereof whose somatic and germline cells contain a stably integrated first recombinant gene encoding an MDE or an enzymatically active derivative or variant thereof, preferably a constitutively active proMMP-13 variant (designated MMP-13*) comprising the sequence depicted in SEQ ID NO: 1. Preferably, the first recombinant gene is under the control of a first regulatable promoter; most preferably, the first regulatable promoter comprises a tet07 sequence, such as, e.g., the promoter depicted in SEQ ID NO: 2. The transgenic animal may further comprise a second recombinant gene encoding a polypeptide that regulates the first regulatable promoter and is preferably a tTA polypeptide. In these embodiments, the second recombinant gene is under the control of a second regulatable promoter, preferably one that comprises sequences derived from a joint-specific promoter, and most preferably a type II collagen promoter, such as, e.g., the promoter depicted in SEQ ID NO: 3. Selective expression of the second recombinant gene in joint tissues thus results in regulated joint-specific expression of the recombinant MDE.

In another aspect, the invention provides isolated nucleic acids encoding enzymatically active MMP variants, preferably human proMMP-13 variants, and most preferably MMP-13*. The invention also encompasses recombinant cloning vectors

comprising these nucleic acids; cells comprising the vectors; methods for producing MMP-13-derived polypeptides comprising culturing the cells under conditions appropriate for MMP-13 expression; and isolated MMP-13-derived polypeptides.

In yet another aspect, the invention provides methods for producing phenotypic changes characteristic of cartilage-degenerative disease in a mammal, which comprise exposing the transgenic animals of the invention to conditions that result in expression of the MDEs encoded by the transgenes. In a preferred embodiment, a transgenic animal comprising a first recombinant gene encoding MMP-13* operably linked to a tet07 promoter and a second recombinant gene encoding a tTA protein operably linked to a type II collagen promoter is maintained in the presence of tetracycline or a tetracycline analogue. When it is desired to induce expression of MMP-13*, tetracycline or the tetracycline analogue is withdrawn, MMP-13* is selectively expressed in joint tissues, and phenotypic changes characteristic of cartilage-degenerative disease result.

In yet another aspect, the invention provides methods for determining the potential of a composition to counteract cartilage-degenerative disease. The methods are carried out by administering a known dose of the composition to the transgenic animals of the invention, either before or after phenotypic indicators of cartilage-degenerative disease have developed; monitoring the indicators for a predetermined time following administration of the composition; and comparing the extent of the indicators in the animal to which the composition was administered relative to a control transgenic animal that had not been exposed to the composition. Any difference in (i) the nature or extent of phenotypic indicators of cartilage-degenerative disease, (ii) the time required for the indicators to

develop, or (iii) the need for other ameliorative treatments indicates the potential of the composition to counteract cartilage-degenerative disease.

Brief Description of the Drawings

Figure 1A is a schematic illustration of the structure of human MMP-13 (collagenase-3). The black box at the extreme aminoterminus represents the pre domain (signal peptide) that targets nascent proMMP-13 for secretion. The lightly hatched box represents the pro domain, which is involved in maintaining the latency of the enzyme. A conserved sequence within the pro domain that is important for maintaining enzyme latency is shown. The heavily hatched box represents the 170-amino acid catalytic domain, which contains a conserved region (shown) that is important for catalytic activity. The shaded box represents the 200-amino acid carboxyterminal domain.

Figure 1B is an illustration of the nucleic acid sequence encoding a constitutively active variant of human pro MMP-13, designated MMP-13*, and the amino acid sequence of MMP-13*, SEQ ID NO:1. The residues that are mutated relative to wild-type MMP-13, which are depicted in larger type, are GTC at nucleotide positions 299-301.

Figures 2A and 2B are schematic illustrations of transgenes used for regulated expression of human MMP-13* in transgenic mice. Figure 2A shows a nucleic acid construct comprising, in a 5' to 3' direction: (i) sequences derived from rat type II collagen promoter; (ii) sequences encoding a tetracycline repressor polypeptide fused in frame to sequences encoding a VP16 transcriptional activator polypeptide; and (iii) sequences comprising an SV40-derived RNA splice site and polyadenylation signal. Figure 2B shows a nucleic acid

construct comprising, in a 5' to 3' direction: (i) sequences derived from a bacterial tet07 promoter; (ii) sequences encoding human MMP-13*; and (iii) sequences comprising an SV40-derived RNA splice site and polyadenylation signal.

Figure 3A is a schematic illustration of a transgene used to assess tissue-specific regulation conferred by a type II collagen promoter. The nucleic acid construct comprises, in a 5' to 3' direction: (i) sequences derived from a rat type II collagen promoter; (ii) sequences encoding bacterial β-galactosidase (LacZ); and (iii) sequences comprising an SV40-derived RNA splice site and polyadenylation signal.

Figure 3B is a color photographic illustration of whole mount staining for β-galactosidase activity of embryonic day 16 transgenic mouse embryos expressing the transgene illustrated in Figure 3A. Blue staining, indicating the presence of enzymatically active B-galactosidase polypeptides, is evident in joints throughout the body of the transgenic animal, while no staining is observed in the non-transgenic, wild-type littermate.

Figure 4 shows color photographic illustrations of immunohistochemical localization of type II collagen cleavage products in the growth plate and articular cartilage of transgenic mice expressing the transgenes shown in Figure 2. The tissues were stained with an antibody that recognizes cleavage products of type II collagen. The left panel shows tissue derived from a mouse that had been maintained on doxycycline to repress MMP-13* expression. The right panel shows tissue derived from a mouse that had been withdrawn from doxycycline, allowing expression of MMP-13*, for 30 days at 3 months of age.

Figure 5 is a color photographic illustration of Safranin O staining of the articular cartilage and growth plate of the patella of double transgenic mice. The left panel shows tissue derived from a mouse maintained on doxycycline. The middle panel shows

tissue derived from a mouse 7 days after withdrawal from doxycycline. The right panel shows tissue derived from a mouse 14 days after withdrawal from doxycycline.

Detailed Description of the Invention

The present inventors have discovered that regulated expression of matrix-degrading enzymes in cartilage in transgenic mice results in characteristic phenotypic changes associated with matrix degenerative diseases of the joints and intervertebral discs. The animal models of the invention provide novel model systems for matrix degenerative disease syndromes which can be used for detailed characterization of human joint and intervertebral disc pathologies as well as for drug discovery and optimization of treatment regimens.

A transgenic animal according to the invention is an animal having cells that contain a transgene which was introduced into the animal or an ancestor of the animal at a prenatal (embryonic) stage. A transgenic animal can be created, for example, by introducing the gene of interest into the male pronucleus of a fertilized oocyte by, e.g., microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The gene of interest may include appropriate promoter sequences, as well as intronic sequences and polyadenylation signal sequences. Methods for producing transgenic animals are disclosed in, e.g., U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan et al., *A Laboratory Manual*, Cold Spring Harbor Laboratory, 1986. A transgenic founder animal can be used to breed additional animals carrying the transgene. A transgenic animal carrying one transgene can also be bred to another transgenic animal carrying a second transgene to create a "double transgenic" animal carrying two transgenes. Alternatively, two transgenes can be co-microinjected to produce a double transgenic animal. Animals carrying more than two

transgenes are also possible. Furthermore, heterozygous transgenic animals, i.e., animals carrying one copy of a transgene, can be bred to a second animal heterozygous for the same transgene to produce homozygous animals carrying two copies of the transgene.

The present invention encompasses transgenic animals, preferably mammals, which express MDEs, particularly MMPs, and most particularly those MMPs having collagenase activity, from a recombinant gene. MDEs for use in the invention include without limitation MMPs and aggrecanase. Useful MMPs include without limitation the collagenases designated MMP-1, MMP-8 and MMP-13; the stromelysins designated MMP-3, MMP-10, and MMP-11; the gelatinases designated MMP-2 and MMP-9; the metalloelastase designated MMP-12; and membrane-type MMPs designated MMP-14, MMP-15, MMP-16, and MMP-17. Matrisian, *BioEssays*, 14:455, 1992. Matrix-degrading activity as used herein refers to the proteolytic degradation of matrix components, including, e.g., collagen, particularly type II collagen and most particularly the triple helical form of type II collagen. Any polypeptide exhibiting matrix-degrading activity may be used in practicing the invention, including enzymatically active fragments of the above-described enzymes. Preferably, MMP-13 enzymatic activity is expressed. MMP-13 enzymatic activity as used herein refers to the proteolytic degradation of type II collagen. Any MMP-13 polypeptide or fragment or derivative thereof that exhibits MMP-13 enzymatic activity may be used. The enzymes may be derived from any animal species, including without limitation human, mouse, rat, rabbit, pig, cow, or non-human primate, or combinations thereof. Preferably, the MMP-13 or derivative thereof is of human origin.

Normally, MMPs are synthesized as precursors (i.e., zymogens or proenzymes) whose enzymatic activity is latent; proteolytic removal of the pro region after secretion

produces the enzymatically active protein. In preferred embodiments of the invention, the need for proteolytic processing is circumvented by the use of enzyme or proenzyme variants that are enzymatically active even when uncleaved. Such variants can be produced using conventional techniques for site-directed or random mutagenesis coupled with analysis of collagenase enzymatic activity (see below). In this manner, modifications (including, e.g., insertions, deletions, and substitutions), may be introduced into a proenzyme sequence, particularly within the pro region or near the pro region cleavage site, to produce a constitutively active polypeptide which does not require proteolytic processing for activation. Alternatively, the pro region may be deleted entirely. Furthermore, recombinant genes may be used in which the sequence encoding the native signal peptide is replaced by a heterologous sequence that functions as a signal peptide, i.e., promotes secretion. The use of genes encoding any such modified MMP polypeptides is encompassed by the invention.

Preferably, a constitutively active MMP-13 variant is used in practicing the invention. Most preferably, the MMP-13 variant comprises a sequence containing a mutation in the sequence encoding the PRCGVPDV region, SEQ ID NO:4, specifically a substitution of Pro^{99} to Val; the sequence of this polypeptide is depicted in SEQ ID NO: 1 and this polypeptide is designated MMP-13*. In another embodiment, the constitutively active MMP-13 variant comprises a substitution of Val^{98} to Gly.

The transgenic animals of the invention preferably express MMP activity in a regulated manner. Regulated expression as used herein refers to temporal and/or spatial control. Temporal control refers to the ability to repress expression of MMP activity until a predetermined time in the development of the transgenic animal, after which MMP expression may be activated and maintained for as long as desired. Preferably, MMP

expression is repressed throughout embryonic development and activated in the adult animal. Spatial control refers to the ability to selectively express MMP activity in particular tissues. Preferably, MMP activity is selectively expressed in joint tissues, most preferably in articular chondrocytes.

Temporal control of MMP expression is achieved by use of one or more polypeptides comprising a transcriptional repressor, a transcriptional activator or enhancer, or combinations thereof, in conjunction with a promoter responsive to the transcriptional repressor/activator used to which the MMP-encoding sequence is operably linked. In one set of embodiments, temporal control of MMP expression is achieved by (i) expression in the transgenic animal of a repressor polypeptide operably linked to a polypeptide that directly or indirectly activates transcription in eucaryotic cells, creating a repressor-activator fusion polypeptide; and (ii) the coupled use of a target promoter operably linked to an MMP-encoding sequence whose transcriptional activity is responsive to the repressor-activator fusion polypeptide. Typically, nucleotide sequences encoding the repressor polypeptide are ligated in-frame to sequences encoding the transcriptional activator polypeptide to create a chimeric gene encoding a fusion protein.

Useful repressor polypeptides include without limitation polypeptides comprising sequences derived from bacterial repressors, including without limitation tetracycline repressor, LacR repressor, KRAB domain, and lambda repressor (cro and cI), as well as eukaryotic repressors, including without limitation those involved in amino acid or sugar synthesis. Useful direct transcriptional activator polypeptides include without limitation herpes simplex virus protein 16 (VP16); yeast GAL14; yeast STAT; steroid receptors such as, e.g., progesterone receptor and estrogen receptor; and constitutive

activators such as, e.g., c-fos, c-jun, and SP-1. Alternatively, the repressor polypeptide may be linked to a polypeptide that indirectly activates transcription by recruiting a transcriptional activator to interact with the repressor-activator fusion protein; such indirect activator polypeptides include without limitation TATA Box Binding Protein (TBP) and basic transcription factors, including, e.g., basic transcription factor D.

According to the invention, each repressor-activator fusion protein is used in conjunction with a target promoter that is responsive to the particular fusion protein and that regulates transcription of an MDE-encoding sequence. Typically, the promoter comprises at least one operator sequence responsive to the repressor component of the repressor-activator fusion polypeptide, which is operably linked to at least a minimal promoter that supports transcription in eucaryotic cells. Examples of suitable repressor-responsive operator sequences include without limitation sequences derived from the tetracycline resistance operon encoded in Tn10 in *E. coli*, the lambda repressor operon, and the yeast GAL repressor operon. Examples of suitable eucaryotic promoters from which minimal promoters may be derived include without limitation the cytomegalovirus (CMV) IE promoter, PtK-1 (thymidine kinase) promoter, HSP (heat shock protein) promoter, and any eukaryotic promoter containing a TATA box. Minimal promoter sequences may be derived from these promoters by (i) creating deletion mutants using conventional methods and (ii) testing the ability of the resulting sequences to activate transcription in a cell line. U.S. Patent No. 5,650,298 discloses a repressor-activator fusion protein comprised of sequences derived from the tetracycline repressor fused to VP16 sequences, which is designated tTA, and a tTA-responsive promoter, designated tet07, which comprises a Tn10-derived sequence linked to a portion of the CMV IE promoter.

Alternatively, temporal control is achieved by (i) expression in the transgenic animal of a heterologous or recombinant transcriptional activator polypeptide or polypeptides and (ii) the coupled use of a target promoter operably linked to an MMP-encoding sequence whose transcriptional activity is responsive to the heterologous or recombinant transcriptional activator. Useful transcriptional activators include without limitation a modified ecdysone receptor, in which a VP16 transactivation domain linked to the aminoterminal transactivation domain of the glucocorticoid receptor is fused to the ligand-binding domain and carboxyterminal sequence of the ecdysone receptor (No et al., *Proc. Natl. Acad. Sci. USA* 93:3346, 1996); a chimeric protein, designated pGL-VP, comprising VP16 activator sequences, GAL4 activation sequences, and a mutated human progesterone receptor ligand-binding domain (Wang et al., *Proc. Natl. Acad. Sci. USA* 91:8180, 1994; Wang et al., *Gene Therapy* 4:432, 1997); and chimeric proteins comprising transcriptional activators fused to estrogen (or other steroid) binding domains (Mattioni et al., *Meth. Cell Biol.* 43:335, 1994). The ecdysone receptor system utilizes retinoid X receptor (RXR) to form heterodimers with the chimeric receptor, and responds to ecdysone, muristerone (an ecdysone analogue) or dexamethasone. The pGL-VP system is responsive to mifepristone (RU486). Chimeric receptors containing an estrogen binding domain respond to hydroxytamoxifen (an estrogen analogue).

Spatial control of MDE expression is achieved by the use of transcriptional promoters that direct transcription selectively in joint tissues. Joint-specific expression as used herein refers to expression that is greater in joints than in other cells; typically, the level of expression in non-joint tissues is less than 10% of the level of expression in joints. Preferably, expression in non-joint tissues is undetectable. Useful promoter sequences that

confer joint-specific expression on a sequence to which they are operably linked include without limitation sequences derived from the collagen type II promoter. It will be understood that a joint-specific promoter according to the invention may comprise one or more copies of particular sequences or sub-sequences, and these sequences may be in direct or inverted orientation relative to each other and relative to the sequence whose expression is regulated by the promoter.

Coordinated spatial and temporal control of MDE expression is preferably achieved by (i) placing expression of the repressor-activator fusion polypeptide or the transcriptional activator polypeptide under the control of a joint-specific promoter; (ii) placing the expression of the MDE or a derivative thereof under the control of a promoter responsive to the repressor-activator fusion polypeptide or the transcriptional activator polypeptide; and (iii) maintaining the transgenic animal during fetal development and early life under conditions in which MDE expression is repressed.

The method by which transgenic animals are maintained during fetal and early post-natal development so that MDE expression is repressed will depend on the particular transgenes being expressed. When a repressor-activator fusion polypeptide is used, repression is achieved by providing the animal with an agent that binds to the repressor-activator fusion protein and results in repression of transcription of the target MDE gene. In animals comprising a transgene encoding a repressor-activator fusion polypeptide containing tet repressor sequences, repression is achieved by providing tetracycline or a tetracycline analogue in the food or drinking water of the mother and, following birth, of the progeny. Tetracycline or an analogue may also be provided using surgically implanted subcutaneous time-release pellets (Innovative Research of America, Inc., Sarasota FL) In this case, binding

of tetracycline or a tetracycline analogue to the repressor-activator fusion protein prevents the fusion protein from binding to, and activating transcription of, the cognate promoter. Tetracycline analogues are compounds closely related to tetracycline which bind to the tet repressor with a Ka of at least about $10^6 M^{-1}$, preferably with an affinity of about $10^9 M^{-1}$ or greater. Useful tetracycline analogues include without limitation doxycycline, anhdryrotetracycline, chlortetracycline, epioxytetracycline, and the like. The dosage used is one that will result in substantial repression of MMP expression. Typically, tetracycline or a tetracycline analogue is administered in the animal's drinking water at a dosage of about 1 mg/ml. When it is desired that MMPs be expressed, the tetracycline or analogue thereof is withheld.

In other embodiments, repression is achieved by withholding from the animal an agent required for activity of the transcriptional activator polypeptide. For example, if the transcriptional activator is a modified ecdysone receptor, the animals are maintained in the absence of ecdysone or an ecdysone analogue throughout fetal and early post-natal development. Ecdysone analogues are compounds closely related to ecdysone which bind to the modified ecdysone receptor with a Ka of at least about $10^6 M^{-1}$. Useful ecdysone analogues include without limitation muristerone A. When it is desired that MDEs be expressed, the animals are given, e.g., ecdysone or muristerone A via intraperitoneal injections at dosages of between about 10 mg and about 20 mg/animal. Similarly, when pGL-VP is used, activation is achieved by providing mifepristone.

In a preferred embodiment of the invention, a transgenic animal is constructed whose somatic and germline cells contain in stably integrated form two recombinant genes: (i) a first recombinant gene comprising a sequence encoding MMP-13*, wherein the sequence

is operably linked to a tetO7 promoter; and (ii) a second recombinant gene encoding a tTA protein operatively linked to a collagen type II promoter. In this embodiment, animals are maintained in the presence of tetracycline or a tetracycline analogue throughout fetal and early post-natal development to repress the gene. Afterwards, tetracycline or the tetracycline analogue is withdrawn, and MMP-13 enzymatic activity is selectively expressed in joint tissues.

Animal Models for Cartilage-Degenerative Diseases

The present invention provides animal model systems in which phenotypic changes characteristic of cartilage-degenerative diseases, such as, e.g., joint or disc disease, are reproducibly exhibited. These diseases include without limitation osteoarthritis, rheumatoid arthritis, chondrodysplasias, and degenerative intervertebral disc diseases. The model systems of the invention exhibit one or more phenotypic indicators common to these diseases, which include without limitation loss of proteoglycan (as indicated by, e.g., loss of Safranin O staining) and cleavage of type II collagen in the affected tissues. The systems encompass the transgenic animals described above, in which recombinant or heterologous MDEs, particularly MMPs, are expressed in cartilage at a predetermined time in the life of the transgenic animal. The timing of the appearance of cartilage-degenerative indicators is determined by activating MDE expression and monitoring the effects on cartilage (see below). Preferably, one or more MDEs are expressed after birth, most preferably after the animal has reached adulthood.

Expression of the transgenes is typically monitored by extracting mRNA from different tissues and subjecting the extracted mRNA to one or more of the following: (i)

reverse transcriptase-polymerase chain reaction (RT-PCR), using primers homologous to the transgene; (ii) RNAase protection; and (iii) Northern blot analysis. Alternatively, in situ hybridization may used.

The physiological effects of MDE expression on articular cartilage are monitored in test animals by sacrificing the animals and subjecting paraffin-embedded decalcified cartilage to staining with (i) hematoxylin and eosin (using conventional techniques) followed by double staining with (ii) Safranin O and fast green. Peter et al., *J. Exp. Pathol.* 71:19, 1990. Alternatively, frozen sections may be obtained and stained with antibodies that are specific for cleavage fragments derived from type II collagen. Billinghurst et al., *J. Clin. Invest.* 99:1534, 1997. Typically, expression of the MMP transgene(s) for at least about 7 days results in detectable loss of proteoglycan and changes in growth plate morphology (see, e.g., Example 5 below). Animal models in which expression of MDEs, particularly MMPs, and most particularly an enzymatically active form of MMP-13, results in proteoglycan loss and/or cleavage of type II collagen are within the scope of the invention.

Other phenotypic indicators of cartilage-degenerative disease which can be monitored in transgenic animals produced according to the invention include without limitation gross observations of changes in joint function and histological evidence of (i) fibrillation and loss of articular cartilage and (ii) osteophyte formation.

Syndromes for which the transgenic animals of the invention provide useful models include without limitation any pathological condition that manifests a disturbance in the composition, morphology, and/or function of cartilage, including osteoarthritis; rheumatoid arthritis; degenerative intervertebral disc diseases; chondrodysplasias, including, e.g., Kniest dysplasia, achondrogenesis, and hypophosphatasia; and proteoglycan-mediated

disorders, such as occur, e.g., in brachymorphic animals. Hall et al., *Cartilage: Molecular Aspects*, CRC Press, 1991, pp. 201-203.

In further embodiments of the invention, the transgenic animals can be subjected to additional treatments to modulate the cartilage-degenerative indicators and/or to supplement the animals' disease phenotype with additional physiological effects such as, e.g., those associated with a particular disease. For example, the transgenic animals may be further treated with inflammatory mediators to augment collagen degradation and/or induce loss of proteoglycan (see, e.g., Example 6 below). Furthermore, the timing and extent of MDE induction, with or without additional treatments, can be adapted to replicate the symptomatology of a particular disease or syndrome.

Methods for Evaluating Drugs that Modulate Degenerative Diseases of Cartilage

The present invention encompasses methods for discovery and evaluation of drugs and therapies for their efficacy against degenerative diseases of cartilage, particularly degenerative joint diseases. In one embodiment of the invention, the transgenic animals of the invention are maintained under conditions in which expression of one or more MDEs results in one or more phenotypic indicators of cartilage-degenerative disease. Once the symptoms have developed, the potential of a composition to counteract cartilage-degenerative disease can be evaluated by administering a known dose of the composition to the animal in which the symptoms have developed; monitoring the phenotypic indicators for a predetermined time following administration of the composition; and comparing the extent of the phenotypic indicators in the animal to which the composition was administered relative to a control animal. Control animals comprise age- and sex-matched transgenic animals that

are maintained under an identical regimen (i.e., express the transgenes) but which do not receive the composition. Any statistically significant difference in the extent or nature of the phenotypic indicators indicates the potential of the composition to counteract cartilage-degenerative disease. As used herein, phenotypic indicators of cartilage-degenerative disease refer to proteoglycan loss, joint space narrowing, collagen degradation, and destruction of cartilage.

In another embodiment of the invention, the potential of a composition to counteract degenerative diseases of cartilage, particularly degenerative joint disease, is evaluated by administering to a transgenic animal a known dose of the composition before and/or simultaneous with the induction of MDE expression in the transgenic animal; monitoring phenotypic indicators of cartilage-degenerative disease for a predetermined time following administration of the composition and MDE induction; and comparing the extent of the phenotypic indicators and/or disease in the animal to which the composition was administered relative to a control animal that had not been exposed to the composition. In this embodiment, any statistically significant difference in the extent or nature of the phenotypic indicators and/or disease, or any statistically significant delay in appearance of the phentoypic indicators or disease, indicates the potential of the composition to counteract cartilage-degenerative disease.

A further indication of the potential of a composition to counteract cartilage-degenerative disease is the ability of the composition to cause any reduction in the extent or duration of other treatments, including, e.g., the dosage and timing of administration of other therapeutic agents used to alleviate symptoms of the disease.

Compounds that may be tested for anti-cartilage-degenerative disease potential may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, synthetic compound libraries, and compounds resulting from directed rational drug design and synthesis. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. Blondelle et al., *TibTech* 14:60, 1996.

Transgenic animals

Transgenic animals as used herein refers to animals into which one or more heterologous and/or recombinant genes have been introduced. The transgenes may be from a different species, or from the same species as the transgenic animal but are not naturally found in the animal in the configuration and/or at the chromosomal locus conferred by the transgene. Transgenes may comprise foreign DNA sequences, i.e., sequences not normally found in the genome of the host animal. Alternatively or additionally, transgenes may comprise endogenous DNA sequences that have been rearranged or mutated *in vitro* in order to alter the normal *in vivo* pattern of expression of the gene, or to alter or eliminate the

biological activity of an endogenous gene product encoded by the gene. Also encompassed by the invention are DNA fragments that are introduced into a pre-existing gene to, e.g., change patterns of expression or to provide additional means of regulating the expression of the gene. Watson et al., "The Introduction of Foreign Genes Into Mice," in *Recombinant DNA*, 2d Ed., W.H. Freeman & Co., New York, 1992, pp. 255-272; Gordon, J.W., *Intl. Rev. Cytol.* 115:171,1989; Jaenisch, *Science* 240:1468, 1989; Rossant, *Neuron* 2:323, 1990.

The transgenic non-human animals of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages are used to introduce the transgenes of the invention. Different methods are used depending on the stage of development of the embryonal target cell(s). Such methods include, but are not limited to, microinjection of zygotes, viral integration, and transformation of embryonic stem cells as described below.

1. Microinjection of zygotes is the preferred method for incorporating transgenes into animal genomes. A zygote, which is a fertilized ovum that has not undergone pronuclei fusion or subsequent cell division, is the preferred target cell for microinjection of transgenic DNA sequences. The murine male pronucleus reaches a size of approximately 20 micrometers in diameter, a feature which allows for the reproducible injection of 1-2 picoliters of a solution containing transgenic DNA sequences. The use of a zygote for introduction of transgenes has the advantage that, in most cases, the injected transgenic DNA sequences will be incorporated into the host animal's genome before the first cell division. Brinster et al., *Proc. Natl. Acad. Sci. USA* 82:4438, 1985. As a consequence, all cells of the resultant transgenic animals (founder animals) stably carry an incorporated transgene at a particular genetic locus, referred to as a transgenic allele. The transgenic allele

demonstrates Mendelian inheritance, i.e., half of the offspring resulting from the cross of a transgenic animal with a non-transgenic animal will inherit the transgenic allele, in accordance with Mendel's rules of random assortment.

2. Viral integration can also be used to introduce the transgenes of the invention into an animal. The developing embryos are cultured *in vitro* to the blastocyte developmental stage. The blastomeres may be infected with appropriate retroviruses. Jaenich, *Proc. Natl. Acad. Sci. USA* 73:1260. Infection of the blastomeres is enhanced by enzymatic removal of the zona pellucida. Transgenes are introduced via viral vectors which are typically replication-defective but which remain competent for integration of viral-associated DNA sequences, including transgenic DNA sequences linked to such viral sequences, into the host animal's genome. Transfection is easily and efficiently obtained by culture of blastomeres on a monolayer of cells producing the transgene-containing viral vector. Alternatively, infection may be performed using cells at a later developmental stage, such as blastocoeles. In any event, most transgenic founder animals produced by viral integration will be mosaics for the transgenic allele; that is, the transgene is incorporated into only a subset of all the cells that form the transgenic founder animals. Moreover, multiple viral integration events may occur in a single founder animal, generating multiple transgenic alleles which will segregate in future generations of offspring. Introduction of transgenes into germline cells by this method is possible but probably occurs at a low frequency. However, once a transgene has been introduced into germline cells by this method, offspring may be produced in which the transgenic allele is present in all of the animal's cells, i.e., in both somatic and germline cells.

3. Embryonal stem (ES) cells can also serve as target cells for introduction of the transgenes of the invention into animals. ES cells are obtained from pre-implantation embryos that are cultured in vitro. Evans et al., *Nature* **292**:154, 1981. ES cells that have been transformed with a transgene can be combined with an animal blastocyst, after which the ES cells colonize the embryo and contribute to the germline of the resulting animal (which is a chimera, i.e., composed of cells derived from two or more animals). Again, once a transgene has been introduced into germline cells by this method, offspring may be produced in which the transgenic allele is present in all of the animal's cells, i.e., in both somatic and germline cells.

Although the initial introduction of a transgene is a Lamarckian (non-Mendelian) event, the transgenes of the invention may be stably integrated into germ line cells and transmitted to offspring of the transgenic animal as Mendelian loci. Other transgenic techniques result in mosaic transgenic animals, in which some cells carry the transgenes and other cells do not. In mosaic transgenic animals in which germ line cells do not carry the transgenes, transmission of the transgenes to offspring does not occur. Nevertheless, mosaic transgenic animals are capable of demonstrating phenotypes associated with the transgenes.

In practicing the invention, animals of the transgenic maintenance line are crossed with animals having a genetic background in which expression of the transgene results in symptoms of cartilage-degenerative disease. Offspring that have inherited the transgenes of the invention are distinguished from littermates that have not inherited transgenes by analysis of genetic material from the offspring for the presence of nucleic acid sequences derived from the transgenes of the invention. For example, biological fluids that contain polypeptides uniquely encoded by the transgenes of the invention may be immunoassayed for the presence

of the polypeptides. A simpler and more reliable means of identifying transgenic offspring comprises obtaining a tissue sample from an extremity of an animal, such as, for example, a tail, and analyzing the sample for the presence of nucleic acid sequences corresponding to the DNA sequence of a unique portion or portions of the transgenes of the invention. The presence of such nucleic acid sequences may be determined by, e.g., hybridization ("Southern") analysis with DNA sequences corresponding to unique portions of the transgene, analysis of the products of PCR reactions using DNA sequences in a sample as substrates, oligonucleotides derived from the transgene's DNA sequence, and the like.

<u>*Nucleic Acids, Vectors, Expression Systems, and Polypeptides*</u>

The present invention encompasses isolated nucleic acids encoding MDEs, particularly MMPs, and enzymatically active fragments derived therefrom, as well as constitutively active MMP variants and enzymatically active fragments derived therefrom. The invention also encompasses complements of the above nucleic acids; vectors comprising the nucleic acids; cells comprising the vectors; and isolated polypeptides encoded by the nucleic acids.

Many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry are used in practicing the present invention, such as those explained in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis,* 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic

Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.).

"Nucleic acid" or "polynucleotide" as used herein refers to purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, such as, for example, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases.

A "coding sequence" or a "protein-coding sequence" is a polynucleotide sequence capable of being transcribed into mRNA and/or capable of being translated into a polypeptide. The boundaries of the coding sequence are typically determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus.

A "complement" of a nucleic acid sequence as used herein refers to the "antisense" sequence that participates in Watson-Crick base-pairing with the original sequence.

An "isolated" nucleic acid or polypeptide as used herein refers to a component that is removed from its original environment (for example, its natural environment if it is naturally occurring). An isolated nucleic acid or polypeptide typically contains less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated.

A nucleic acid or polypeptide sequence that is "derived from" a designated sequence refers to a sequence that corresponds to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are homologous or complementary

to the sequence, as well as "sequence-conservative variants" and "function-conservative variants." For polypeptide sequences, this encompasses "function-conservative variants." Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in a polypeptide has been changed without altering the overall conformation and function of the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). "Function-conservative" variants also include any polypeptides that have the ability to elicit antibodies specific to a designated polypeptide.

Nucleic acids comprising any of the sequences disclosed herein or subsequences thereof can be prepared by conventional methods. For example, DNA can be chemically synthesized using, e.g., the phosphoramidite solid support method of Matteucci *et al.*, 1981, *J. Am. Chem. Soc.* **103**:3185, the method of Yoo *et al.*, 1989, *J. Biol. Chem.* **764**:17078, or other well known methods. This can be performed by sequentially linking a series of oligonucleotide cassettes comprising pairs of synthetic oligonucleotides.

Due to the degeneracy of the genetic code, many different nucleotide sequences can encode polypeptides having the amino acid sequences defined herein or subsequences thereof. The codons can be selected for optimal expression in prokaryotic or eukaryotic systems. Such degenerate variants are also encompassed by this invention.

The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such

as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. PNAs are also encompassed by the term "nucleic acid". The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The polypeptides of the invention may be expressed by using many known vectors, such as pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP plasmids (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the practice of the invention. Recombinant cloning vectors will often include one or more replication systems for cloning or expression; one or more markers for selection in the host, such as, for example, antibiotic resistance; and one or more expression cassettes. The inserted coding sequences may be synthesized by standard methods, isolated from natural sources, prepared as hybrids, or the like. Ligation of the coding sequences to transcriptional regulatory elements and/or to other amino acid coding sequences may be achieved by known methods. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including

electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, yeast, plant, and animal cells, and especially mammalian cells. Of particular interest are *E. coli, S. aureus, B. subtilis, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Schizosaccharomyces pombi, SF9* cells, C129 cells, 293 cells, *Neurospora*, CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, cytomegalovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and are effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art (Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley, 1997). Under appropriate expression conditions, host cells can be used as a source of recombinantly produced peptides and polypeptides.

The MDEs of the present invention, including function-conservative variants, may be isolated from native or heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which the protein-coding sequence has been introduced and expressed. Alternatively, these polypeptides may be produced in cell-free protein synthesis systems, which may additionally be supplemented with microsomal membranes to achieve glycosylation and signal peptide processing of preprocollagenases. Furthermore, the polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation, or classical solution synthesis.

Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The construction and analysis of MMP variants and derivatives that exhibit enzymatic activity, and preferably constitutive enzymatic activity, can be achieved by routine application of conventional methods. First, a nucleic acid encoding an MMP is modified either by site-directed or random mutagenesis, or is used in a construction scheme as one segment of a fusion gene. Preferably, the procedure results in a modification either contained within the sequence encoding the pro region or near the pro region cleavage site; this includes deleting the pro region entirely. Alternatively, sequences may be constructed that encode fusion proteins either between enzymatically active MMP domains and other polypeptides, or between different MMPs. The modified nucleic acid is then used to program synthesis of a variant MMP, either in a cell-free system, in intact cells (including permeabilized cells), or in a transgenic animal. Preferably, either a cell-free system or a cell culture system is used to express the MMP variant or derivative. The extent of pro region cleavage is assessed by metabolic labelling and resolution of the MMP product by SDS-PAGE. Finally, MMP enzymatic activity is measured using conventional assays, such as, by quantifying the

cleavage of natural substrates or model peptides, as disclosed, e.g., in Weingarten et al., *Biochem.* 24:6730, 1985; Woessner et al., *J. Biol. Chem.*, 263:16918, 1988, and Knight et al., *FEBS Letts.*, 296:263, 1992. In this manner, a large number of MMP variants and derivatives, including, e.g., function-conservative variants of MMP-13*, can be created routinely and assayed for MMP enzymatic activity.

Description of the Preferred Embodiments

The following examples are intended to illustrate the present invention without limitation.

Example 1: Construction of a Gene Encoding a Modified, Constitutively Active ProMMP-13

The following experiments were performed to create a gene encoding a procollagenase derived from MMP-13 that is enzymatically active in the absence of pro region cleavage. The sequence of this proMMP-13 variant, designated MMP13*, is shown in Figure 1B, SEQ ID NO:1.

Site-directed mutagenesis was performed to modify MMP-13 cDNA as follows:

A cDNA fragment encoding proMMP was obtained by digesting plasmid pNot3A (Freije et al., *J. Biol. Chem.* 269:16766, 1994; GENBANK accession number X75308) with XbaI and HindIII and purifying the resulting ~1515 bp fragment. This fragment was subcloned into the Tet-resistant/Amp-sensitive pAlter plasmid (Promega, Madison, WI) that had been digested with XbaI and HindIII.

Site-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI). Briefly, phagemid single-stranded DNA was purified from cultures containing the helper phage R408 (Promega). In addition to the Amp repair - Tet knock-out conversion oligos (Promega), an oligonucleotide having the sequence 5'-AAGCCAAGATGCGGGGTTGTCGATGTGGGTGAATACAAT-3', SEQ ID NO:5, was phosphorylated and annealed to the single-stranded DNA, followed by mutant strand synthesis. The reaction mixture was then used to transform the repair-minus *E. coli* strain

ES1301 *mutS*, and the culture was grown in ampicillin selective media. Plasmid DNA was isolated from isolated clones and transformed into JM109 cells, which were then plated on LB plates containing 120 μg/ml ampicillin.

The above procedure resulted in a proline-to-valine substitution at amino acid 99. The modified proMMP was designated MMP13* (Figure 1B, SEQ ID NO:1).

Using a similar technique, site-directed mutagenesis was also used to introduce a valine to glycine mutation at amino acid 98. A mutagenic oligonucleotide having the sequence 5'-GAAAAAGCCAAGATGCGGGGTCCTGATGTGGGTGAATAC-5', SEQ ID NO:6 was used as described above. This procedure resulted in a valine-to-glycine substitution at amino acid 98.

After confirmation of the above mutations by direct sequencing, cDNA encoding MMP13* cDNA was excised from the pAlter vector by digestion with Eco RI and Hind III.

The enzymatic activity of MMP-13* was determined as follows:

1. cDNAs encoding both mutant forms of MMP13 and wild-type MMP-13 were subcloned into a BS(SK-) vector (Stratagene) containing the CMV promoter (Xho I - Eco RI) and the SV40 splice poly (A)n (Xba I - Nco I). Duplicate cultures of Hela cells (10 cm dishes) were transfected with 50 μg of these plasmids using the $CaPO_4$ precipitation method (Promega). Five hours later, cells were subjected to a 1-minute glycerol shock using a solution containing an equal volume of 2 X HBS + 30 % glycerol. This procedure is described in the Profection Mammalian Transfection Systems technical manual (Promega).

2. Twenty-four hours following transfection, the culture medium (D-MEM containing 10 % fetal bovine serum) was replaced with D-MEM containing no serum and 10

μM CGS-27023A (Ciba-Geigy), an MMP inhibitor. It is believed that, in the absence of an added MMP inhibitor, MMPs produced by the culture autodigest; thus, addition of an MMP inhibitor to the culture medium resulted in a detectable MMP13 band.

3. Forty-eight hours after the addition of serum-free medium containing the MMP inhibitor, 10 ml of supernatant were collected and concentrated about 200-fold using Centriprep-30 and Centricon-10 concentrators (Amicon), after which an equal volume of 2X Tris-glycine SDS running buffer was added to each sample. The samples were then applied to a 4-16% pre-stained beta-casein zymogram SDS polyacrylamide gel (Novex). After electrophoresis, the gels were renatured in renaturing buffer (Novex) for 30 minutes at room temperature, followed by overnight incubation at 37°C in zymogram developing buffer (Novex).

The results indicated that cells expressing either a variant MMP13 containing a proline -> valine substitution at position 99 (MMP-13*) or a variant MMP13 containing a valine -> glycine substitution at position 98 secreted detectable MMP activity similar to cells expressing wild-type MMP-13. This method thus provides a rapid screen for MMP13 variants that retain MMP13 enzymatic activity.

In a further step, cDNA encoding MMP-13* was operably linked to a transcriptional regulatory sequence derived from the tet07 promoter as follows:

1. The BS(SK-) vector (Stratagene) was digested with KpnI and NotI. A synthetic duplex oligonucleotide having the following sequence was digested with KpnI and Not I and ligated to the vector:

5'-GGTACCACTAGTAAGCTTAGATCTCATATGGTCGACCCCGGGGAATTCCTGCAGGGATCCTCTAGAAGTACTCCATGGGTATACATCGATGCGGCCGC-3', SEQ ID NO:7

The SB(SK-) vector as modified above was digested with XbaI and NcoI. A 745 bp fragment containing the SV40 splice site and polyadenylation signal, which was obtained by digesting pcDNAI/Amp (Invitrogen, Carlsbad, CA) with XbaI and NcoI, was ligated to this vector. This 745 bp fragment was recovered by digestion of the vector with XbaI and NotI and was inserted into the original BS(SK-) vector.

2. The resulting vector was linearized by digestion with XhoI and EcoRI and ligated to a 460 bp XhoI-EcoRI fragment containing the tetO7 promoter region (Gossen et al., *Proc. Natl. Acad. Sci. USA* 89:5547, 1992). This vector was then digested with SpeI, blunt-ended with Klenow polymerase, and digested with EcoRI.

3. pAlter-MMP13* was digested with HindIII, blunt-ended with Klenow polymerase, and digested with EcoRI to obtain an MMP13*-encoding fragment.

4. The MMP13* EcoRI fragment was cloned into the EcoRI digested vector obtained in step 2.

5. The 2792 bp transgene, SEQ ID NO:8, was excised by digestion with XhoI and NotI and purified using CsCl gradient centrifugation.

Example 2: Construction of a Collagen Type II-Promoter-Linked tTa gene

The following experiments were performed to create a gene encoding a tTA repressor-activator fusion protein operably linked to a joint-specific (type II collagen)

promoter as well as a reporter gene suitable for assessing the tissue-specific expression conferred by the type II collagen promoter.

1. *Type II collagen promoter- tetracycline/VP16 transgene:* The modified BS(SK-) vector containing the SV40 splice site and polyadenylation signal as described in Example 1 above was digested with NdeI and Sma I and ligated to a 1897 bp fragment containing the collagen II promoter and enhancer. This fragment was obtained by digesting plasmid PBS$\underline{\Delta}$H1 with HindIII, after which it was blunt-ended with Klenow and digested with NdeI.

The plasmid was then digested with EcoRI and BamHI and ligated to a 1025 bp fragment encoding the tetracycline/VP16 repressor-activator fusion protein that had been excised from the pUHG15-1 plasmid (Gossen et al., *Proc. Natl. Acad. Sci. USA* <u>89</u>:5547, 1992) using EcoRI and BamHI. The plasmid was linearized by digestion with BglII, dephosphorylated using calf intestinal phosphatase, and ligated to a 1554 BamHI enhancer fragment obtained from plasmid PBS$\underline{\Delta}$H1.

Finally, the 5276 bp transgene, SEQ ID NO:9, was excised from the vector by digestion with KpnI and NotI, gel purified, purified by CsCl gradient centrifugation, dialyzed against microinjection buffer (5 mM Tris-HCl pH 7.4, 0.1 mM EDTA pH 8.0) and used for microinjection (see Example 3 below).

2. *Type II collagen promoter-β-galactosidase gene*: A 4179 bp BamHI-BglII fragment containing the β-galactosidase gene fused to the β-globin splice sequence and polyadenylation signal was excised from plasmid pUGH16-3 (Gossen et al., *Proc. Natl. Acad. Sci. USA* <u>89</u>:5547, 1992) and cloned into the BamHI site of unmodified BS(SK-) (Stratagene). This plasmid was digested with EcoRI and HindIII and ligated to a 655 bp Hind III-Eco RI

fragment containing the type II collagen promoter sequence, which was excised from the plasmid described in (1) above. The plasmid was then digested with EcoRI and ligated to a 2807 bp Eco RI fragment which had been excised from the type II collagen promoter plasmid described above. Restriction mapping was used to verify the orientation of each insert. The 7664 bp transgene, SEQ ID NO:10, was excised by digestion with HindIII and NotI, gel purified, purified by CsCl gradient centrifugation, dialyzed against microinjection buffer (5 mM Tris pH 7.4, 0.1 mM EDTA pH 8.0), and microinjected into mouse embryos.

Example 3: Production and Characterization of Transgenic Mice Expressing Tetracycline-Regulated MMP-13 in Joint Tissues

The following experiments were performed to produce transgenic mice expressing MMP-13* or a LacZ (β-galactosidase) reporter gene.

To produce mice expressing MMP-13* under tetracycline regulation, an XhoI-NotI tet07-MMP-13* DNA fragment (Figure 2B, SEQ ID NO:8) and a KpnI - NotI CPE-tTA DNA fragment (Figure 2A, SEQ ID NO:9) were co-microinjected into fertilized mouse embryos in equimolar amounts. To produce mice expressing the reporter gene, a HindIII-NotI LacZ fragment (described in Example 2 above) was injected into (FVB/N) fertilized eggs as described (Hogan et al., *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratories, 1996).

Founder animals were first identified by PCR as follows. The tTA-encoding transgene was identified using a primer corresponding to the tTA sequence (5'-CGAGGGCCTGCTCGATCTCC-3', SEQ ID NO:11) and a primer corresponding to a 3' untranslated sequence (5'-GGCATTCCACCACTGCTCCC-3', SEQ ID NO:12). The

resulting PCR product was 584 bp in size. The MMP13* -encoding transgene was identified using primers corresponding to sequences encoding MMP13* (5'-GAGCACCCTTCTCATGACCTC-3', SEQ ID NO:13) and the 3' untranslated region, respectively. The resulting PCR product was 731 bp in size. Out of 112 newborn mice, 7 transgenic founders harboring both transgenes were found.

The LacZ-encoding transgene was identified using primers corresponding to the nuclear localization signal of the β-galactosidase gene (5'-GTTGGTGTAGATGGGCGCATCG-3', SEQ ID NO:14) and the collagen promoter (5'-GCGGGGTCTCAGGTTACAGCC-3', SEQ ID NO:15). The resulting PCR product was 673 bp in size.

Southern blot analysis of tail DNA digested with BamHI/NcoI or PvuII/NcoI and hybridized to the 3' untranslated region under high stringency conditions was performed to confirm the results obtained using PCR. The number of copies of transgene DNA that integrated into the genome was determined by comparing the relative intensity of the hybridization signal from transgenic mice with that obtained using control DNAs containing 10 and 100 genome equivalents of the same DNA that was injected. Transgenic lines were generated by mating founder animals to FVB/N wild type animals.

All mice were administered doxycycline (Sigma Chemical Co., St. Louis MO) at a concentration of 1.0 mg/ml in acidic drinking water, which was changed on a daily basis. Schultze et al., *Nature Biotech.* 14:499, 1996.

Example 4: Analysis of Joint-Specific Expression Conferred by Type II Collagen Promoter Constructs

The following experiments were performed to evaluate tissue-specific expression conferred by use of the type II collagen promoter.

Joint-specific expression was monitored by staining for β-galactosidase activity as described (Hogan et al., *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratories, 1996); using this method, the presence of enzymatically active β-galactosidase is reflected in the appearance of a blue stain.

Wild-type female mice were mated with transgenic males harboring the CPE-LacZ construct as described in Examples 2 and 3 above. On embryonic day 16, the females were sacrificed, and the embryos were stained for β-galactosidase activity. Prior to fixation, the tails were removed from the embryos and used as a source of template DNA for PCR reactions to determine transgene transmission.

Figure 3B illustrates the blue staining that is observed in joints throughout the body of the embryonic day 16 transgenic mouse embryo. Specifically, β-galactosidase expression was observed in ankles, knees, hips, phalanges, wrists, elbows, shoulders, and vertebrae. In addition to the cartilage of the joints, cartilage that had not ossified to bone at this stage of development, *i.e.,* some of the facial, skull, and rib bones, also expressed β-galactosidase. No staining was observed in non-transgenic, wild type littermates.

These results indicated that the type II collagen-derived promoter according to the present invention is capable of conferring joint-specific expression on sequences to which it is operably linked.

Example 5: Analysis of the Phenotypic Effects of Joint-Specific Expression of MMP-13*

The following experiments were performed to evaluate the development of phenotypic indicators of cartilage degeneration in transgenic animals expressing MMP-13* in joint tissue.

Mice harboring both the tet07-MMP-13* and CPE-tTA constructs (produced as described in Example 3 above) were maintained on doxycyline until adulthood (approximately 8 weeks postpartum).

Expression of MMP13* was first evaluated in hemizygous mice using RT-PCR to detect the transcripts. No expression of the transgene was observed in any of the lines in any of the tissues sampled, including brain, heart, liver, kidney, hindlimb, muscle, bone, or tongue.

To examine whether MMP-13* DNA in any of the double-transgenic lines was capable of being expressed, embryonic fibroblasts were prepared from these animals and transfected with a tTA expression plasmid (Gossen and Bujard, *Proc.Natl.Acad.Sci.USA* 89:5547, 1992). The transfection was done because the joint-specific type II collagen promoter regulating tTA expression (and thereby MMP-13* expression) in the transgenic animals might not be expected to function in embryonic fibroblasts.

For this purpose, wild-type females were mated to transgenic males harboring both the type II collagen promoter-linked tTA and Tet07-MMP-13* transgenes. On embryonic day 15, the females were sacrificed, and fibroblasts were prepared from the embryos (Graham et al., *Virol.* 52:456, 1973; Lopata et al., *Nuc. Acid Res.* 12:5707, 1984). The fibroblasts were cultured in DMEM containing 10% fetal bovine serum, and were transfected with the tTa expression plasmid using the calcium phosphate method. Forty-eight

hours after transfection, total RNA was prepared from the cells using the Trizol method (GIBCO/BRL, Grand Island, NY). RT-PCR was performed using the Superscript preamplification system (GIBCO/BRL) for first-strand cDNA, after which MMP-13* sequences were detected by PCR using the following MMP-13*-specific primers: 5'-GCCCTCTGGCCTGCTGGCTCATG-3', SEQ ID NO:16 and 5'-CAGGAGAGTCTTGCCTGTATCCTC-3', SEQ ID NO:17.

Fibroblasts from several transgenic lines (such as, e.g., lines 8 and 42) were capable of expressing MMP13*, as evidenced by the appearance of a PCR product of the predicted size. No MMP13* RT-PCR band was detected from cells transfected with vehicle alone. These results indicated that, in these mice, the MMP13* transgene is integrated into a transcriptionally active region of the chromatin.

Expression of MMP13* in the double transgenic lines was further analyzed by immunohistochemistry, using antibodies specific for MMP-13-derived type II collagen cleavage fragments. For this purpose, joints were fixed in 4% paraformaldehyde in PBS at neutral pH for 60 minutes at room temperature. They were then rinsed twice in PBS, incubated in 0.1M Tris-HCl, pH 7.4, overnight, and partially decalcified in 0.2M EDTA at neutral pH. The samples were transferred to TOC medium and 6-mm frozen sections were obtained using a Hacker/Bright cryostat. The sections were stained with an antibody that recognizes an epitope present in a degradation product of type II collagen, specifically, in the TC^A degradation product, which is also designated the 3/4 piece. Billinghurst et al., *J. Clin. Invest.* <u>99</u>:1534, 1997.

As early as 3 days after removal of the mice from doxycycline, MMP-13 cleavage products could be detected. After 30 days without doxycycline, a substantial increase in

staining in the growth plate and in the articular cartilage could be seen (Figure 4), but the results differed among different lines of mice (see Table 1 below).

Table 1

Immunohistochemistry				
F1 Animal	Days off Dox	hMMP13 Ab	Type II Collagen Cleavage Fragments Ab	Loss of Safranin O Stain
Line 8	wt	-	-	not remarkable
Line 8	0 d	-	-	" "
	3 d	+	+	" "
	7 d	++	++	Mild
	14 d	+++	+++	Moderate
	wt	-	-	not remarkable
Line 6	30 d			Moderate
Line 8	30 d	+++	+++	Moderate
Line 42	30 d	+	-	not remarkable

To study the effect of MMP13* activity on cartilage in adult transgenic animals, mice were withdrawn from doxycycline for increasing times, after which they were sacrificed. Paraffin-embedded formaldehyde-fixed sections of decalcified cartilage were sectioned and stained with (i) hematoxylin and eosin and (ii) Safranin O followed by fast green (American Histo Labs, Gaithersburg MD). Peter et al., *J. Exp. Pathol.* 71:19, 1990.

Control transgenic animals that lack MMP13* expression retain a significant amount of safranin O stain in both the articular cartilage as well as the growth plate of their patella (Figure 5, left panel). By contrast, transgenic animals from line 8 show a substantial loss of safranin O staining in their joints following doxycyline withdrawal. After seven days,

a mild reduction of safranin O staining is observed in the articular cartilage of the patella (Figure 5, middle panel), which progresses by day 14 to moderate loss of stain in articular cartilage as well as the growth plate (Figure 5, right panel). A significant loss of safranin O stain was also observed in the other joints including the cartilage of the tarsus and femur, as well as wrist and knuckle, indicating a reduced proteoglycan concentration in these areas compared to controls.

Example 6: Augmentation of the Development of Symptoms of Joint Degenerative Disease in MMP-13 Transgenic Mice

The following treatments are performed to enhance the symptoms of joint degeneration exhibited by the transgenic animals of the invention.

A group of transgenic mice are treated to induce expression of the transgenes at 4-12 weeks of age. Two to six weeks after induction, the mice are injected intraperitonealy with an inflammatory agent, including without limitation, lipopolysaccharide (10-100 μg), zymosan (1-10 mg), the superantigen Staphylococcal Enterotoxin B (1-100 μg), or TGF-β (1-10 μg). Alternatively, the animals are injected intraarticularly with an inflammatory or chondrocyte function-modulating agent, including without limitation, lipopolysaccharide (1-100 ng), zymosan (50-250 μg), papain (10-100 μg), TGF-β (0.01-1 μg), Bone Morphogenic Protein -2 (2-1000 ng), IL-1 (1-100 ng), TGF-α (10-200 ng), IGF (0.01-1 μg), or FGF (0.01-1 μg). Age- and sex-matched transgenic mice maintained under a regimen in which the transgenes are not expressed receive the same treatment and serve as controls.

The development of symptoms of degenerative joint disease is monitored by gross observation of joint swelling and function, and by histological evaluation of the joint at selected timepoints after exposure to the inflammatory agent.

The agents will induce an acute inflammatory response and/or transient loss of proteoglycan with a duration of less than one week. The acute inflammatory response and/or transient cartilage changes will upregulate gene expression in the chondrocytes, enhancing the expression of the transgene and increasing the levels of MMP-13 produced.

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference in their entirety.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

Claims:

1. A transgenic non-human mammal or the progeny thereof having somatic and germline cells which contain, in stably integrated form, a recombinant gene encoding a polypeptide comprising an enzymatically active matrix-degrading enzyme, wherein said recombinant gene is selectively expressed in chondrocytes of said mammal and said expression results in pathological symptoms characteristic of cartilage-degenerative disease.

2. A transgenic animal or the progeny thereof having somatic and germline cells which contain a stably integrated first recombinant gene encoding a polypeptide selected from the group consisting of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16, MMP-17 and enzymatically active variants thereof.

3. A transgenic animal as defined in claim 2, wherein said first recombinant gene is selectively expressed in synovial chondrocytes of said animal.

4. A transgenic animal as defined in claim 2, wherein said first recombinant gene encodes MMP-13.

5. A transgenic animal as defined in claim 4, wherein said MMP-13 is human.

6. A transgenic animal as defined in claim 4, wherein said first recombinant gene encodes a variant MMP-13 polypeptide comprising enzymatically active proMMP-13.

7. A transgenic animal as defined in claim 6, wherein said recombinant gene comprises a MMP-13-encoding sequence as depicted in SEQ ID NO:1.

8. A transgenic animal as defined in claim 2, wherein said animal is selected from the group consisting of mouse, rat, rabbit, sheep, cow, goat, and pig.

9. A transgenic animal as defined in claim 8, wherein said animal is a mouse.

10. A transgenic animal as defined in claim 2, wherein expression of said recombinant gene is under the control of a first regulatable promoter.

11. A transgenic animal as defined in claim 10, wherein said first regulatable promoter comprises tetO7.

12. A transgenic animal as defined in claim 11, wherein said promoter has the sequence depicted in SEQ ID NO:2.

13. A transgenic animal as defined in claim 10, further comprising a second recombinant gene encoding a polypeptide that regulates said first promoter.

14. A transgenic animal as defined in claim 12, wherein expression of said second recombinant gene is under the control of a second regulatable promoter.

15. A transgenic animal as defined in claim 13, wherein said second regulatable promoter comprises sequences derived from a type II collagen promoter that confer selective expression of said second recombinant gene in joint tissues.

16. A transgenic mouse or the progeny thereof having somatic and germline cells which contain in stably integrated form:

(i) a first recombinant gene comprising a sequence encoding a variant MMP-13 polypeptide comprising MMP-13*, wherein said sequence is operably linked to a tetO7 promoter; and

(ii) a second recombinant gene encoding a tTA protein operatively linked to sequences derived from a type II collagen promoter.

17. A transgenic mouse as defined in claim 16 wherein expression of said recombinant genes in joint tissue results in pathological symptoms characteristic of joint degenerative disease.

18. An isolated nucleic acid encoding enzymatically active proMMP-13, wherein said nucleic acid has a sequence selected from the group consisting of the sequence depicted in SEQ ID NO:1, sequence-conservative mutants thereof, and function-conservative mutants thereof.

19. A recombinant cloning vector comprising a nucleic acid as defined in claim 18.

20. A host cell comprising a vector as defined in claim 19.

21. A method for producing a polypeptide comprising culturing a cell as defined in claim 20 under conditions appropriate for expression of said enzymatically active proMMP-13.

22. A method for producing phenotypic changes associated with cartilage-degenerative disease in a mammal, comprising maintaining a mammal as defined in claim 1 under conditions in which said recombinant gene is selectively expressed in joint tissue of said mammal.

23. A method for producing phenotypic changes associated with cartilage-degenerative disease in a mammal, comprising maintaining a mammal as defined in claim 2 under conditions in which said recombinant gene is selectively expressed in joint tissue of said mammal.

24. A method for producing phenotypic changes associated with cartilage-degenerative disease in a mouse, comprising maintaining a mouse as defined in claim 16 for a predetermined time in the absence of tetracycline or biologically active analogues thereof.

25. A method for determining the potential of a composition to counteract cartilage-degenerative disease, said method comprising:

(i) administering a known dose of the composition to a transgenic animal as defined in claim 1 under conditions in which phenotypic indicators associated with cartilage-degenerative disease are expressed;

(ii) monitoring development of the phenotypic indicators of cartilage-degenerative disease for a predetermined time following administration of the composition; and

(iii) comparing the extent of the phenotypic indicators in the animal to which the composition was administered relative to a control transgenic animal that had not been exposed to the composition,

wherein any difference in the nature or extent of the phenotypic indicators, or any difference in the time required for the phenotypic indicators to develop, indicates the potential of the composition to counteract cartilage-degenerative disease.

26. A method for determining the potential of a composition to counteract cartilage-degenerative disease, said method comprising:

(i) maintaining a transgenic animal as defined in claim 16 for a predetermined time in the absence of tetracycline or a tetracycline analogue, wherein said maintainence results in the appearance of one or more phenotypic indicators of cartilage-degenerative disease in said animal;

(ii) administering a known dose of said composition to the animal;

(iii) monitoring development of said phenotypic indicators for a predetermined time following administration of the composition; and

(iii) comparing the extent of the phenotypic indicators in the animal to which the composition was administered relative to a control transgenic animal that had not been exposed to the composition,

wherein any difference in the nature or extent of the phenotypic indicators indicates the potential of the composition to counteract cartilage-degenerative disease.

27. A method for determining the potential of a composition to counteract cartilage-degenerative disease, said method comprising:

(i) providing a transgenic animal as defined in claim 16 that had been maintained in the presence of tetracycline or a tetracycline analogue to repress expression of said first recombinant gene;

(ii) substantially simultaneously (a) administering to said animal a known dose of said composition and (b) withdrawing said tetracycline;

(iii) monitoring development of phenotypic indicators of cartilage-degenerative disease for a predetermined time following administration of the composition; and

(iv) comparing the extent of the phenotypic indicators in the animal to which the composition was administered relative to a control transgenic animal that had not been exposed to the composition,

wherein any difference in the nature or extent of the indicators, or any difference in the time required for the indicators to develop, indicates the potential of the composition to counteract cartilage-degenerative disease.

ABSTRACT OF THE DISCLOSURE

The present invention provides animal model systems for cartilage-degenerative disease, which comprise transgenic animals which can express recombinant matrix-degrading enzymes (MDEs), particularly matrix metalloproteinases (MMPs), in a temporally and spatially regulated manner. The invention also provides methods for producing phenotypic indicators of cartilage-degenerative disease in a mammal and methods for determining the potential of a composition to counteract cartilage-degenerative disease. The invention also provides isolated nucleic acids encoding proMMP polypeptides that exhibit constitutive enzymatic activity and isolated proMMP polypeptides.

A

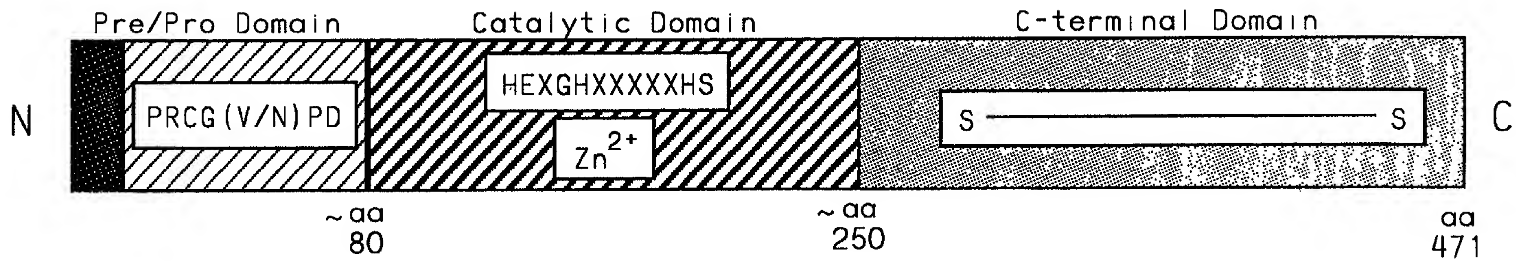

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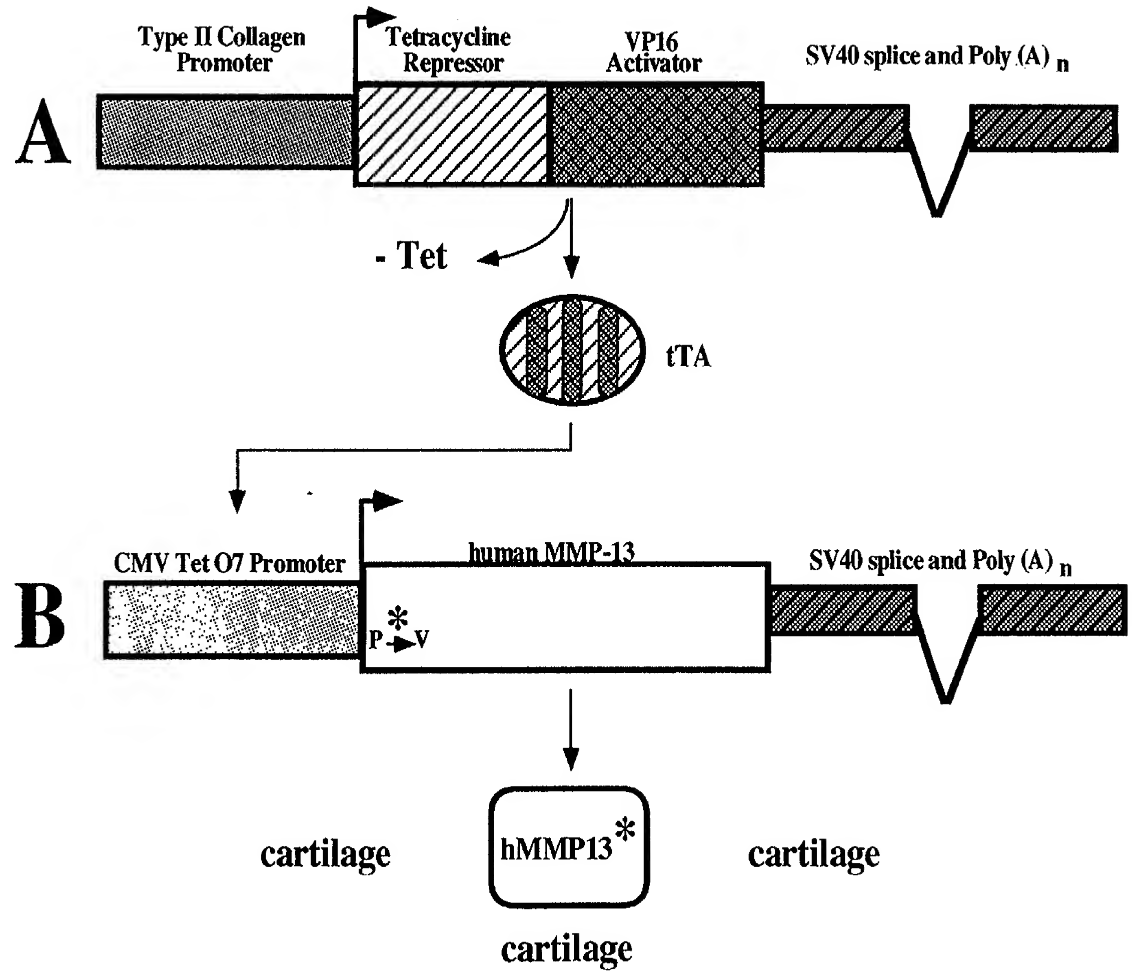
Figure 1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tTA
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CMV Tet O7 Promoter
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P*V
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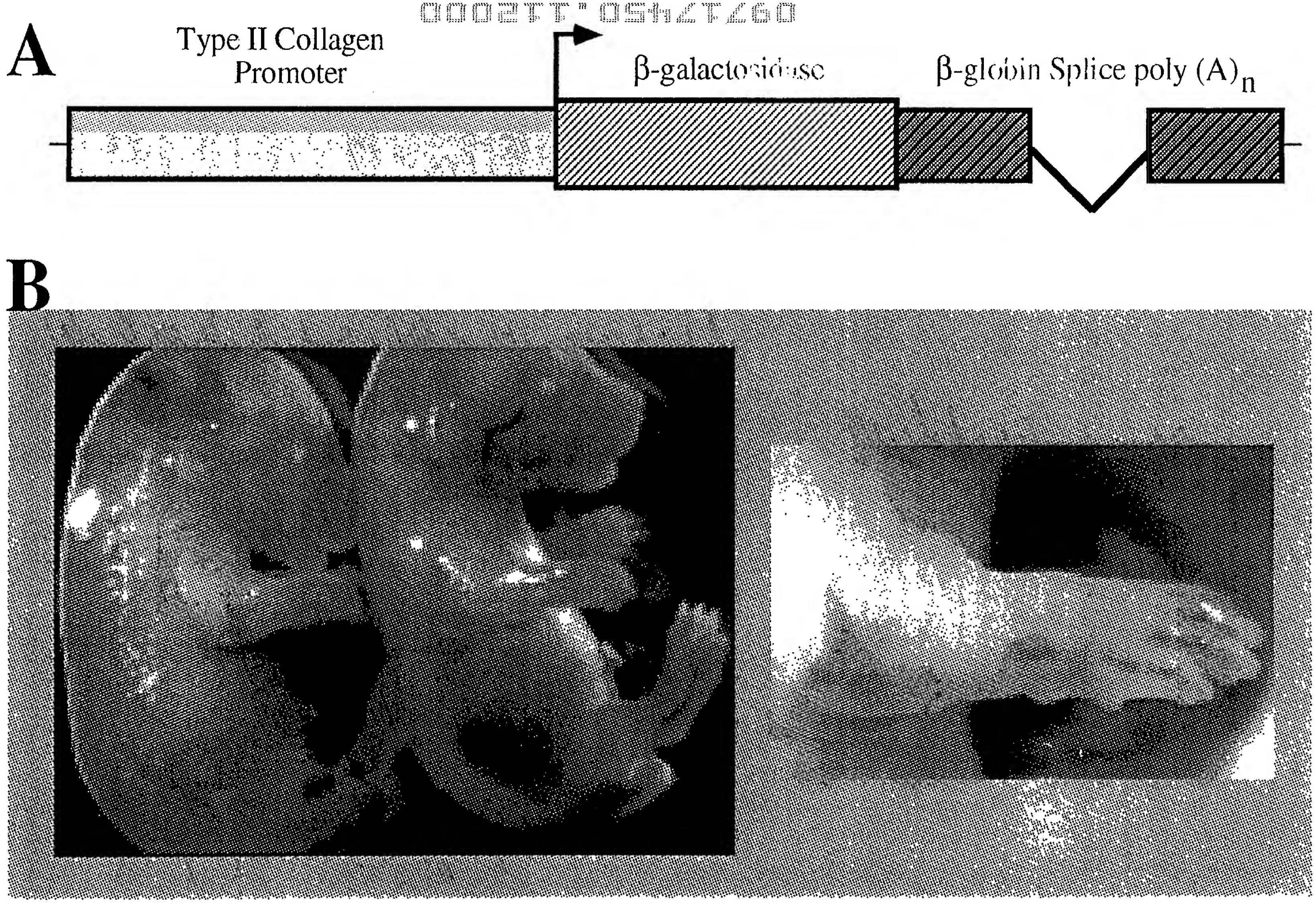
hMMP13*
cartilage
cartilage
cartilage

Figure 2



Transgenic **Wildtype** **Elbow & Front Paw**

Figure 3

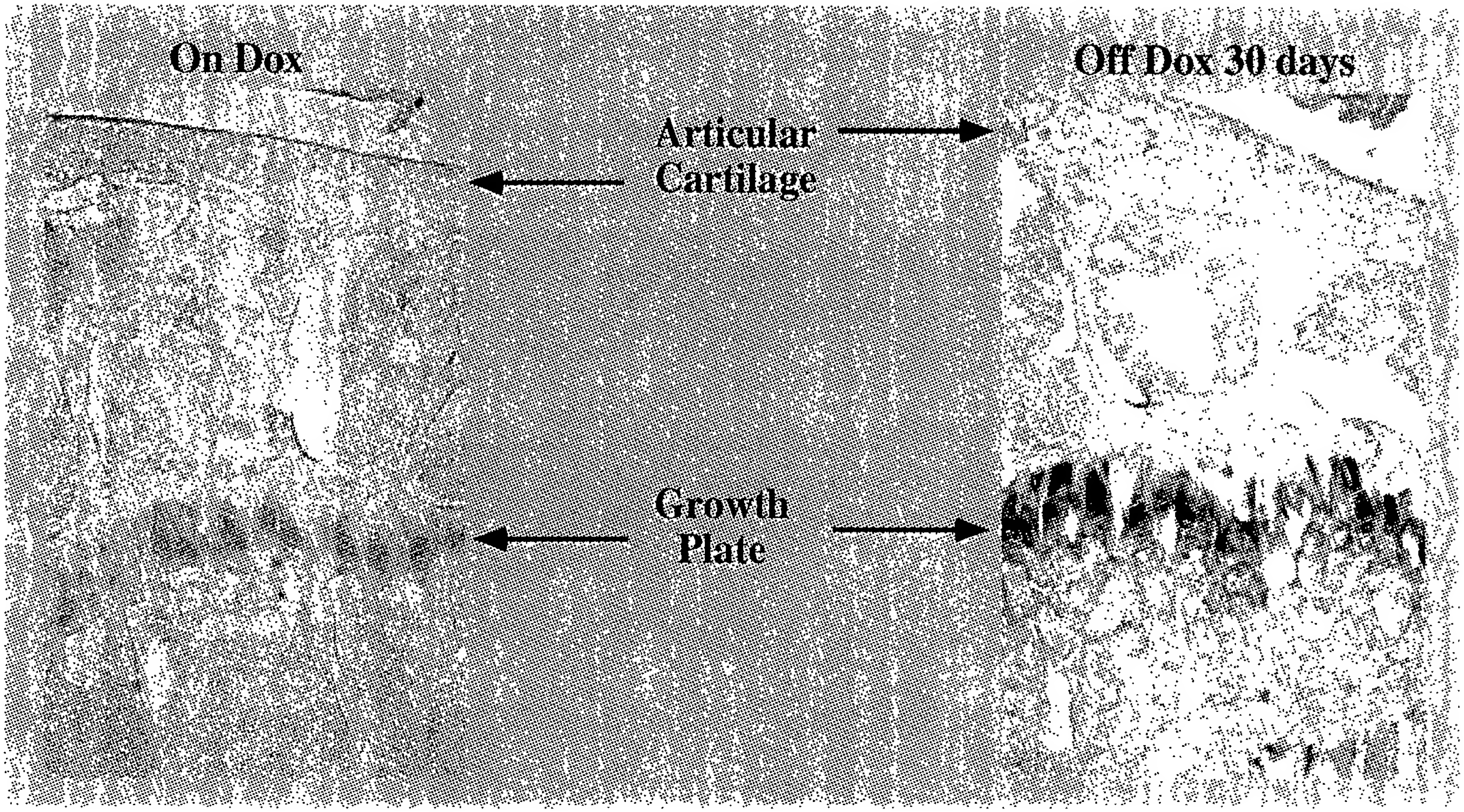


Figure 4

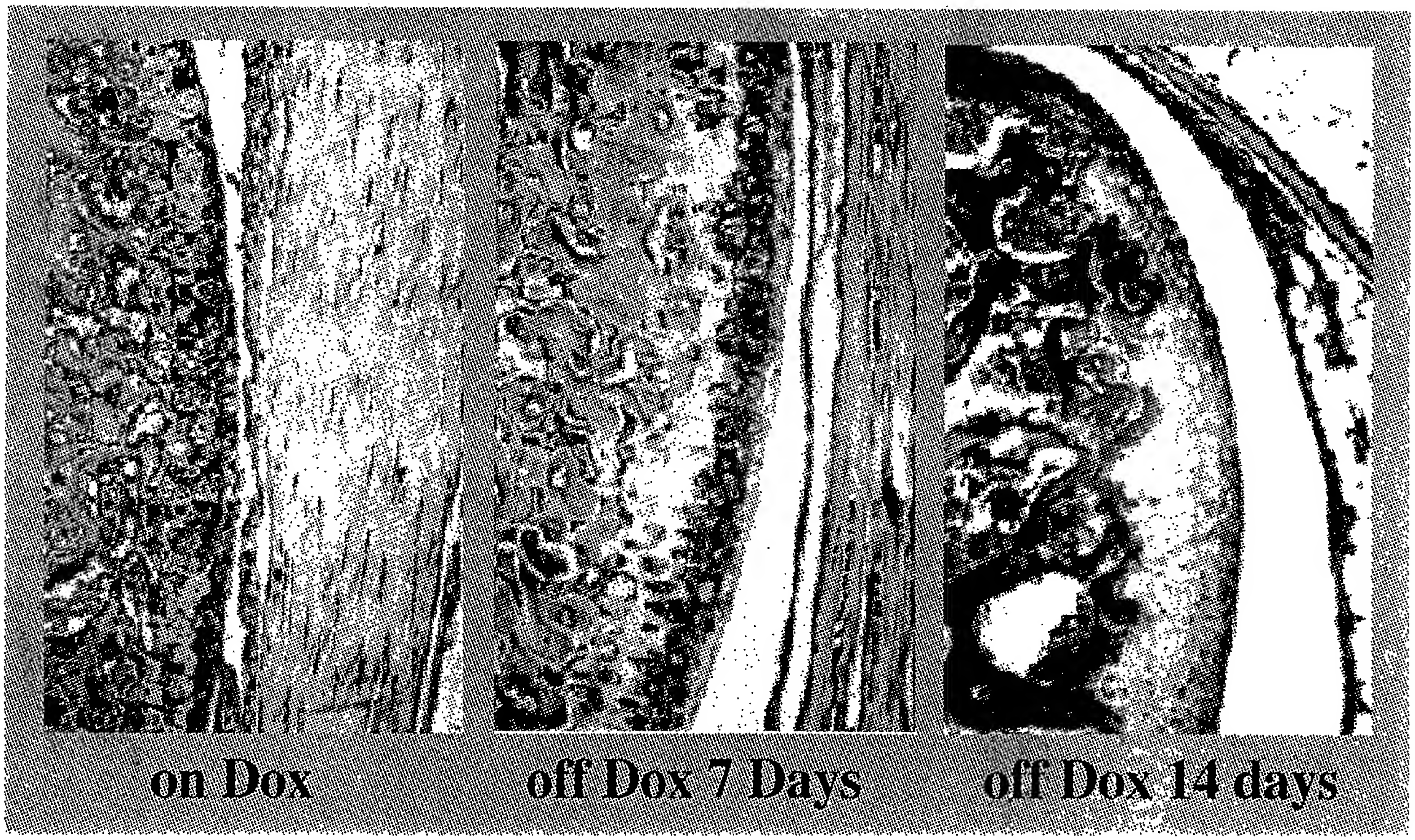


Figure 5

FILE NO.: 0630/0D532

DECLARATION
AND POWER OF ATTORNEY
Original Application

As a below named inventor, I declare that the information given herein is true, that I believe that I am the original, first and sole inventor if only one name is listed at 1 below, or a joint inventor if plural inventors are named below, of the invention entitled:

TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

which is described and claimed in the attached specification,

that I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application, that I acknowledge my duty to disclose information of which I am aware which is material to patentability in accordance with 37 CFR §1.56. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I hereby claim the priority benefits under 35 U.S.C. 119 of any application(s) for patent or inventor's certificate listed below. All foreign applications for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns prior to the application(s) of which priority is claimed are also identified below.

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agents(s) to prosecute this application and transact all business in the Patent and Trademark office connected therewith:

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CITY: Newtown STATE OR FOREIGN COUNTRY: Pennsylvania COUNTRY OF CITIZENSHIP: USA

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STATE OR COUNTRY: Pennsylvania ZIP CODE: 18940

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 1: Lisa A Nalbad DATED: 12/18/97

United States of America)
State of: N.J.) S.S.:
County of: Middlesex)

MARIE ARCARO
MY COMMISSION EXPIRES Marie Arcaro
ON JUNE 9, 1999

Sworn to and subscribed before me this 18th day of December, 1997.

SIGNATURE OF INVENTOR 2: Lora Marie Keller DATED: 12/18/97

United States of America)
State of: N.J.) S.S.:
County of: Middlesex)

Sworn to and subscribed before me this 18th day of December, 1997.

MARIE ARCARO
MY COMMISSION EXPIRES
ON JUNE 9, 1999

Marie Arcaro

(D&DForms/PTO-21)
EV. 12/87

M:\0630\0D532\LC3949

09/214750

SEQUENCE LISTING

(1) GENERAL INFORMATION

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Killar, Loran

(ii) TITLE OF THE INVENTION: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 10022

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:08/994,689
(B) FILING DATE: 1997-12-19
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Green, Reza
(B) REGISTRATION NUMBER: 38,475
(C) REFERENCE/DOCKET NUMBER: 0630/0D532

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-527-7700
(B) TELEFAX: 212-753-6237
(C) TELEX: 236687

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 471 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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65                  70                  75                  80
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(2) INFORMATION FOR SEQ ID NO:2:

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(A) LENGTH: 470 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3479 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AAAGTGTATT AGGTAGAGCA TTTTCTTCCA CGTGTTTGGG CACGTTTCCG ACAGCTAGGA 120
TTCCAGCTCT GTCTTTGTAT GTTACAGACT GTAAATCAAT CGCAGGTGAA ACTGTTTGGA 180
CAGTAGGTGG GGATCAAAGA CCCTCCGCCC GTGAGACTCT AGGCGCTTTC CCCTGCCACC 240
AGCCTGTCTC CAGAGATGCT CTGGAAGGAG GCGGGCCCGG GCGGTCTTTC TGCTCTTTAG 300
CGTGGCGGAC GCGGCGGCGG GGGCAGGGCT GGAGCAGAGA GCGCTGCAGT GATAGAACTT 360
TCTGACCCCG CTGCGCAGGG CGGCAGGGTG GCAGGGTGGC AGGGTGGCGA GCTAAGCCAG 420
AGCCGAACGC TGGAGCTCTG GGAGGAACAT CGAAGGTTTG TATGTGGTCT GAGATCGGCC 480
TGACTATATT TTTTTGTCCT AAATTTGCAA GCACACACCC ACAAAGCTGC GGTCTTGACC 540
GGTATTCTTT ATAGAGCGCA ATGGAGTGAG CTGAGTGTCT AAACGATTTC CCTAATTCAT 600
CTGATAGCAG AGGCGCTCTC CTAATTGGCG AAGAGCTGCC TCATGTCCGC AACTTTTTGG 660
CAGAGTGAAT TCCACAGCTT TGTGTGTGTG TGTGGGGGGG GGTGTAAGGG GTGTCTAAAA 720
CTTTCGGTCT CCTACTATTC TGTATCTCGA CCGGTTGGTT TTACACCCCG GCTCATCTCA 780
TCAACGCAAA CACCCCCACT CTCCTATGGA CCCAAGGACC TGACGTGGGG GAAGGTGGAC 840
ATTAGGAATG TCAGAAACCT AGAGTCCACG CTCCTCCTCT CCATCTTTCC ACGAGTTTGG 900
GAAACTTCTT GGCTGCGAAG ACTTTGACCC ACATCTGCAT TTCTCAGCCC CAGCTTCCAA 960
AAGTGCTGCA GGTTCGGGAG GGAGACCTC AGTCCTCCTT TGTGAGGCTT GTTTGCGTTG 1020
AGGGATTGGC AGCGATGGCT TCCAGATGGG CTGAAACCCT GCCCGTATTT ATTTAAACTG 1080
GTTCCTCGTG GAGAGCTGTG AATCGGGCTC TGTATGCGCT CGAGAAAAGC CCCATTCATG 1140
AGAGGCAAGG CCCAGTGGGT CCCCCCGACT CCCCGACCCC CCTCTCCCAC AATATATCCC 1200
CCCTCCCTGT GCCCGCCTGC CGCCACCTCC CGGGCTCCGG CCCCGCGCGC AGCGGCGACG 1260
AAGCAACACA GTTCCCCGAA AGAGGTAGCT TTTTAATTGG CCAGCCACAA AGAATCACTT 1320
ATGCCGCACG GCGGTAACGA GGGGAACCGG ATCGGGCGGC CAGGATGCTA TCTGTGTAGC 1380
CCTTTTCGTG CCACAATTAG GGTGGTGCTG GCTTCCTCCG ACCGCACCTA GGCGATCTGG 1440
TTACACTGTT GGCTCCTTTC TTGGGCAGTC ATTTAATCCT ACTTTTTACT CTACGAATGT 1500
CTGTCTGATG GAGGGCTGTG TCCGGAGCCC CATCCACAAA GAGTCAGCCA GCAGCTCTCA 1560
CACCCGGCTG GATCTCATAT GGTGCACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT 1620
AAGCCAGCCA AGCTAGCTTG CGCAAGCTAG CTTGCGATCC GTAAAAATGT GTGAGAGTTA 1680
CAAAATGTCT TCCGGGCTAA GATCCGACAG CCATGGTCCA AAGAAGACTT CGGCACTGCA 1740
GACTTAAAAC CAGCTTTCTA GCAGAGGCAG AAGGATCTAG AGCCAAAGGC AAAGACTTGA 1800
ATAGGCTGGG AAGATGCAAG AATGGCATTT TACATAAAGA ACACTCTCTC CTTTTCCAGC 1860
CAGCACACTT GCATAGAAAT TAAGTTTTAC ACTTGAAGTT CTTTGTTTCC ATCCTGAGAA 1920
GCTCCAAAGT CTGAGGTGGT GTGGTATGCT GGGTAATTCT CCCCACCCCC CAACATTCCC 1980
TGGGGGTTCC ATGGGGGTAG CTTCTCCCAA GGACTTCCAG CGGCAACACA GAAATCCCAC 2040
TTCGAGACAA AGGAGTTACT GCTTAAATCA GGCCCTAATT TCCAAGGTTC CCTTTGCTTA 2100
AAGTTCCCTA GAGGACCATC TCACTTCTAA AGAAAAGGTG TATTCGGGGA CCCATCCTCA 2160
ACCTCCTTGT TATGGAAGGA GACTTCGGGA ACAGAGCAAG GGCTGAGCCT CCGGCAGTTT 2220
GGGGTAAGGT TGGGGTTGGG GGGAGCAAGG AAGGCAAGTG AGGCTGGAGG CCCAGGGATA 2280
GGGGAAGATG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTC TCGGGGATGG 2340
TGGTGGTGGA CAACTAGGAA ACTCTGGCGC TTTCTCCTCC CCTCACAAAA CTGAGTCCAG 2400
CTGGAGCCGC CTCCAGACTC TCTGGCCAGG GCCTCAGAGT GGTCAACAGT CCCTGGCCAG 2460
CGTTGCTCTC TCCAGGCTAA GGGCACCCAC TCCCCTGGAG ATTCCTGAAC CTGGGCCAGG 2520
AAGAGCCGAA TTAGACAAGT GTCTCCAATC CGGCTGCGTG CGGATTTTGT TGCGGTGTCC 2580
CTCGGTTGTC TGCAGTTCCT TTAGTCCCTT CCCTGGCCTG CCCCTTACAC CTCCACACAG 2640
GTCCCCCTCT GTGTAGGAAT ACACCAGACC CTCTCTTAGC CACACACACC TCCAGTCCCC 2700
CGTCTACCTA GATTTTTTTC ATAGCTAGTT GGATGGGGGA TGGGTTAGGG AGGCTGGGTT 2760
TGCGAGCCTC CAGGTGGGAG TTCACCGACA GGTACTCCGC AAAGGAGCTG GAAGGCAGGT 2820
CTGGAAAACT GTCCCCCAGA TTTAGGATTC TGGGCAGCTT CCATCAGCTT ATACTTTGGC 2880
TCCCCCGCCC CCTAAACTCC CCATCCCCAC CTTCCTTTCT CCCGTTACTT CGTCCTCCCT 2940
CGCCTTTCCA GCCTTGAGTC TAAAGCTCCA TGCTTATGCC TCTGCAAACA ACCCCCTCCC 3000
TTCTAACCCC AGCAGAACTC CGAGGAAAGG GGCCGGAGGC CCCCCTTCTC GCCTGTGGTT 3060
AGAGGGGGCA GTGTGGCAGT CCCAAGTGGG GGCGACCGGA GGCCGTCTCG GTGCCCCGCC 3120

CGATCAGGCC ACTGGGCACA TCGGGGGCGG GAAGCTGGGC TCACCAAAGG GGCGACTGGC 3180
CTTGGCAGGT GTGGGCTCTG GTCCGGCCTG GGCAGGCTCC GGGGGCGGGG TCTCAGGTTA 3240
CAGCCCCGCG GGGGGCTGGG GGGCGGCCCG CGGTTTGGGC TGGTTTGCCA GCCTTTGGAG 3300
CGACCGGGAG CATATAACCG GAGCCTCTGC TGGGAGAAGA CGCAGAGCGC CGCTGGGCTG 3360
CCGGGTCTCC TGCCTCCTCC TCCTGCTCCT AGAGCCTCCT GCATGAGGGC GCGGTAGAGA 3420
CCCGGACCCG CTCCGTGCTC TGCCGCCTCG CCGAGCTTCG CCCGCAAGCT GGGGAATTC 3479

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Arg Cys Gly Val Pro Asp Val
1   5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGCCAAGAT GCGGGGTTGT CGATGTGGGT GAATACAAT 39

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAAAAAGCCA AGATGCGGGG GTCCTGATGT GGGTGAATAC 40

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GGTACCACTA GTAAGCTTAG ATCTCATATG GTCGACCCCG GGGAATTCCT GCAGGGATCC     60
TCTAGAAGTA CTCCATGGGT ATACATCGAT GCGGCCGC                             98
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2792 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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CTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT TACCACTCCC     60
TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACTC CCTATCAGTG ATAGAGAAAA    120
GTGAAAGTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC    180
ACTCCCTATC AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG    240
AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG    300
CTCGGTACCC GGGTCGAGTA GGCGTGTACG GTGGGAGGCC TATATAAGCA GAGCTCGTTT    360
AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC ATAGAAGACA    420
CCGGGACCGA TCCAGCCTCC GCGGCCCCGA ATTAGCTTGA TATCGAATTC GAGCTCGGTA    480
CCCGGGGATC CTCTAGACAA GATGCATCCA GGGGTCCTGG CTGCCTTCCT CTTCTTGAGC    540
TGGACTCATT GTCGGGCCCT GCCCCTTCCC AGTGGTGGTG ATGAAGATGA TTTGTCTGAG    600
GAAGACCTCC AGTTTGCAGA GCGCTACCTG AGATCATACT ACCATCCTAC AAATCTCGCG    660
GGAATCCTGA AGGAGAATGC AGCAAGCTCC ATGACTGAGA GGCTCCGAGA AATGCAGTCT    720
TTCTTCGGCT TAGAGGTGAC TGGCAAACTT GACGATAACA CCTTAGATGT CATGAAAAAG    780
CCAAGATGCG GGGTTGTCGA TGTGGGTGAA TACAATGTTT TCCCTCGAAC TCTTAAATGG    840
TCCAAAATGA ATTTAACCTA CAGAATTGTG AATTACACCC CTGATATGAC TCATTCTGAA    900
GTCGAAAAGG CATTCAAAAA AGCCTTCAAA GTTTGGTCCG ATGTAACTCC TCTGAATTTT    960
ACCAGACTTC ACGATGGCAT TGCTGACATC ATGATCTCTT TTGGAATTAA GGAGCATGGC   1020
GACTTCTACC CATTTGATGG GCCCTCTGGC CTGCTGGCTC ATGCTTTTCC TCCTGGGCCA   1080
AATTATGGAG GAGATGCCCA TTTTGATGAT GATGAAACCT GGACAAGTAG TTCCAAAGGC   1140
TACAACTTGT TTCTTGTTGC TGCGCATGAG TTCGGCCACT CCTTAGGTCT TGACCACTCC   1200
AAGGACCCTG GAGCACTCAT GTTTCCTATC TACACCTACA CCGGCAAAAG CCACTTTATG   1260
CTTCCTGATG ACGATGTACA AGGGATCCAG TCTCTCTATG GTCCAGGAGA TGAAGACCCC   1320
AACCCTAAAC ATCCAAAAAC GCCAGACAAA TGTGACCCTT CCTTATCCCT TGATGCCATT   1380
ACCAGTCTCC GAGGAGAAAC AATGATCTTT AAAGACAGAT TCTTCTGGCG CCTGCATCCT   1440
CAGCAGGTTG ATGCGGAGCT GTTTTTAACG AAATCATTTT GGCCAGAACT TCCCAACCGT   1500
ATTGATGCTG CATATGAGCA CCCTTCTCAT GACCTCATCT TCATCTTCAG AGGTAGAAAA   1560
TTTTGGGCTC TTAATGGTTA TGACATTCTG GAAGGTTATC CCAAAAAAAT ATCTGAACTG   1620
GGTCTTCCAA AAGAAGTTAA GAAGATAAGT GCAGCTGTTC ACTTTGAGGA TACAGGCAAG   1680
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ACTCTCCTGT TCTCAGGAAA CCAGGTCTGG AGATATGATG ATACTAACCA TATTATGGAT 1740
AAAGACTATC CGAGACTAAT AGAAGAAGAC TTCCCAGGAA TTGGTGATAA AGTAGATGCT 1800
GTCTATGAGA AAAATGGTTA TATCTATTTT TTCAACGGAC CCATACAGTT TGAATACAGC 1860
ATCTGGAGTA ACCGTATTGT TCGCGTCATG CCAGCAAATT CCATTTTGTG GTGTTAAGTG 1920
TCTTTTTAAA AATTGTTATT TAAATCCTGA AGAGCATTTG GGGTAATACT TCCAGAAGTG 1980
CGGGGTAGGG GAAGAAGAGC TATCAGGAGA AAGCTCTAGT TCTAGAGGGC CCTATTCTAT 2040
AGTGTCACCT AAATGCTAGA GGATCTTTGT GAAGGAACCT TACTTCTGTG GTGTGACATA 2100
ATTGGACAAA CTACCTACAG AGATTTAAAG CTCTAAGGTA AATATAAAAT TTTTAAGTGT 2160
ATAATGTGTT AAACTACTGA TTCTAATTGT TTGTGTATTT TAGATTCCAA CCTATGGAAC 2220
TGATGAATGG GAGCAGTGGT GGAATGCCTT TAATGAGGAA AACCTGTTTT GCTCAGAAGA 2280
AATGCCATCT AGTGATGATG AGGCTACTGC TGACTCTCAA CATTCTACTC CTCCAAAAAA 2340
GAAGAGAAAG GTAGAAGACC CCAAGGACTT TCCTTCAGAA TTGCTAAGTT TTTTGAGTCA 2400
TGCTGTGTTT AGTAATAGAA CTCTTGCTTG CTTTGCTATT TACACCACAA AGGAAAAAGC 2460
TGCACTGCTA TACAAGAAAA TTATGGAAAA ATATTTGATG TATAGTGCCT TGACTAGAGA 2520
TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC TTTAAAAAAC CTCCCACACC 2580
TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG TTTATTGCAG 2640
CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT 2700
CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCA 2760
TCCCGCCATG GGTATACATC GATGCGGCCG CC 2792

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5276 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTACCACTA GTAAGCTTAG ATCCACTGTC TGGGATTATA TCAGGACAAC CGAAGCCTGG 60
AAAGTGTATT AGGTAGAGCA TTTTCTTCCA CGTGTTTGGG CACGTTTCCG ACAGCTAGGA 120
TTCCAGCTCT GTCTTTGTAT GTTACAGACT GTAAATCAAT CGCAGGTGAA ACTGTTTGGA 180
CAGTAGGTGG GGATCAAAGA CCCTCCGCCC GTGAGACTCT AGGCGCTTTC CCCTGCCACC 240
AGCCTGTCTC CAGAGATGCT CTGGAAGGAG GCGGGCCCGG GCGGTCTTTC TGCTCTTTAG 300
CGTGGCGGAC GCGGCGGCGG GGGCAGGGCT GGAGCAGAGA GCGCTGCAGT GATAGAACTT 360
TCTGACCCCG CTGCGCAGGG CGGCAGGGTG GCAGGGTGGC AGGGTGGCGA GCTAAGCCAG 420
AGCCGAACGC TGGAGCTCTG GGAGGAACAT CGAAGTGTTT GTATGTGGTC TGAGATCGGC 480
CTGACTATAT TTTTTTGTCC TAAATTTGCA AGCACACACC CACAAAGCTG CGGTCTTGAC 540
CGGTATTCTT TATAGAGCGC AATGGAGTGA GCTGAGTGTC TAAACGATTT CCCTAATTCA 600
TCTGATAGCA GAGGCGCTCT CCTAATTGGC GAAGAGCTGC CTCATGTCCG CAACTTTTTG 660
GCAGAGTGAA TTCCACAGCT TTGTGTGTGT GTGTGGGGGG GGGTGTAAGG GGTGTCTAAA 720
ACTTTCGGTC TCCTACTATT CTGTATCTCG ACCGGTTGGT TTTACACCCC GGCTCATCTC 780
ATCAACGCAA ACACCCCCAC TCTCCTATGG ACCCAAGGAC CTGACGTGGG GGAAGGTGGA 840
CATTAGGAAT GTCAGAAACC TAGAGTCCAC GCTCCTCCTC TCCATCTTTC CACGAGTTTG 900
GGAAACTTCT TGGCTGCGAA GACTTTGACC CACATCTGCA TTTCTCAGCC CCAGCTTCCA 960
AAAGTGCTGC AGGTTCGGGA GGGGAGACCT CAGTCCTCCT TTGTGAGGCT TGTTTGCGTT 1020
GAGGGATTGG CAGCGATGGC TTCCAGATGG GCTGAAACCC TGCCCGTATT TATTTAAACT 1080
GGTTCCTCGT GGAGAGCTGT GAATCGGGCT CTGTATGCGC TCGAGAAAAG CCCCATTCAT 1140

GAGAGGCAAG GCCCAGTGGG TCCCCCCGAC TCCCCGACCC CCCTCTCCCA CAATATATCC 1200
CCCCTCCCTG TGCCCGCCTG CCGCCACCTC CCGGGCTCCG GCCCCGCGCG CAGCGGCGAC 1260
GAAGCAACAC AGTTCCCCGA AAGAGGTAGC TTTTTAATTG GCCAGCCACA AAGAATCACT 1320
TATGCCGCAC GGCGGTAACG AGGGGAACCG GATCGGGCGG CCAGGATGCT ATCTGTGTAG 1380
CCCTTTTCGT GCCACAATTA GGGTGGTGCT GGCTTCCTCC GACCGCACCT AGGCGATCTG 1440
GTTACACTGT TGGCTCCTTT CTTGGGCAGT CATTTAATCC TACTTTTTAC TCTACGAATG 1500
TCTGTCTGAT GGAGGGCTGT GTCCGGAGCC CCATCCACAA AGAGTCAGCC AGCAGCTCTC 1560
ACACCCGGCT GGATCTCATA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT 1620
TAAGCCAGCC AAGCTAGCTT GCGCAAGCTA GCTTGCGATC CGTAAAAATG TGTGAGAGTT 1680
ACAAAATGTC TTCCGGGCTA AGATCCGACA GCCATGGTCC AAAGAAGACT TCGGCACTGC 1740
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AATAGGCTGG GAAGATGCAA GAATGGCATT TTACATAAAG AACACTCTCT CCTTTTCCAG 1860
CCAGCACACT TGCATAGAAA TTAAGTTTTA CACTTGAAGT TCTTTGTTTC CATCCTGAGA 1920
AGCTCCAAAG TCTGAGGTGG TGTGGTATGC TGGGTAATTC TCCCCACCCC CCAACATTCC 1980
CTGGGGGTTC CATGGGGGTA GCTTCTCCCA AGGACTTCCA GCGGCAACAC AGAAATCCCA 2040
CTTCGAGACA AAGGAGTTAC TGCTTAAATC AGGCCCTAAT TTCCAAGGTT CCCTTTGCTT 2100
AAAGTTCCCT AGAGGACCAT CTCACTTCTA AAGAAAAGGT GTATTCGGGG ACCCATCCTC 2160
AACCTCCTTG TTATGGAAGG AGACTTCGGG AACAGAGCAA GGGCTGAGCC TCCGGCAGTT 2220
TGGGGTAAGG TTGGGGTTGG GGGGAGCAAG GAAGGCAAGT GAGGCTGGAG GCCCAGGGAT 2280
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GCGTTGCTCT CTCCAGGCTA AGGGCACCCA CTCCCCTGGA GATTCCTGAA CCTGGGCCAG 2520
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TCTGGAAAAC TGTCCCCCAG ATTTAGGATT CTGGGCAGCT TCCATCAGCT TATACTTTGG 2880
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TCGCCTTTCC AGCCTTGAGT CTAAAGCTCC ATGCTTATGC CTCTGCAAAC AACCCCCTCC 3000
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ACCCGGACCC GCTCCGTGCT CTGCCGCCTC GCCGAGCTTC GCCCGCAAGC TGGGGAATTC 3480
ATATGTCTAG ATTAGATAAA AGTAAAGTGA TTAACAGCGC ATTAGAGCTG CTTAATGAGG 3540
TCGGAATCGA AGGTTTAACA ACCCGTAAAC TCGCCCAGAA GCTAGGTGTA GAGCAGCCTA 3600
CATTGTATTG GCATGTAAAA AATAAGCGGG CTTTGCTCGA CGCCTTAGCC ATTGAGATGT 3660
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GTAATAACGC TAAAAGTTTT AGATGTGCTT TACTAAGTCA TCGCGATGGA GCAAAAGTAC 3780
ATTTAGGTAC ACGGCCTACA GAAAAACAGT ATGAAACTCT CGAAAATCAA TTAGCCTTTT 3840
TATGCCAACA AGGTTTTTCA CTAGAGAATG CATTATATGC ACTCAGCGCT GTGGGGCATT 3900
TTACTTTAGG TTGCGTATTG GAAGATCAAG AGCATCAAGT CGCTAAAGAA GAAAGGGAAA 3960
CACCTACTAC TGATAGTATG CCGCCATTAT TACGACAAGC TATCGAATTA TTTGATCACC 4020
AAGGTGCAGA GCCAGCCTTC TTATTCGGCC TTGAATTGAT CATATGCGGA TTAGAAAAAC 4080
AACTTAAATG TGAAAGTGGG TCCGCGTACA GCCGCGCGCG TACGAAAAAC AATTACGGGT 4140
CTACCATCGA GGGCCTGCTC GATCTCCCGG ACGACGACGC CCCCGAAGAG GCGGGGCTGG 4200

CGGCTCCGCG CCTGTCCTTT CTCCCCGCGG GACACACGCG CAGACTGTCG ACGGCCCCCC 4260
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TATTTTAGAT TCCAACCTAT GGAACTGATG AATGGGAGCA GTGGTGGAAT GCCTTTAATG 4740
AGGAAAACCT GTTTTGCTCA GAAGAAATGC CATCTAGTGA TGATGAGGCT ACTGCTGACT 4800
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CAGAATTGCT AAGTTTTTTG AGTCATGCTG TGTTTAGTAA TAGAACTCTT GCTTGCTTTG 4920
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TGATGTATAG TGCCTTGACT AGAGATCATA ATCAGCCATA CCACATTTGT AGAGGTTTTA 5040
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GTTGTTGTTA ACTTGTTTAT TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA 5160
AATTTCACAA ATAAAGCATT TTTTTCACTG CATTCTAGTT GTGGTTTGTC CAAACTCATC 5220
AATGTATCTT ATCATGTCTG GATCATCCCG CCATGGGTAT ACATCGATGC GGCCGC 5276

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7664 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTACCACTA GTAAGCTTAG ATCCACTGTC TGGGATTATA TCAGGACAAC CGAAGCCTGG 60
AAAGTGTATT AGGTAGAGCA TTTTCTTCCA CGTGTTTGGG CACGTTTCCG ACAGCTAGGA 120
TTCCAGCTCT GTCTTTGTAT GTTACAGACT GTAAATCAAT CGCAGGTGAA ACTGTTTGGA 180
CAGTAGGTGG GGATCAAAGA CCCTCCGCCC GTGAGACTCT AGGCGCTTTC CCCTGCCACC 240
AGCCTGTCTC CAGAGATGCT CTGGAAGGAG GCGGGCCCGG GCGGTCTTTC TGCTCTTTAG 300
CGTGGCGGAC GCGGCGGCGG GGGCAGGGCT GGAGCAGAGA GCGCTGCAGT GATAGAACTT 360
TCTGACCCCG CTGCGCAGGG CGGCAGGGTG GCAGGGTGGC AGGGTGGCGA GCTAAGCCAG 420
AGCCGAACGC TGGAGCTCTG GGAGGAACAT CGAAGTGTTT GTATGTGGTC TGAGATCGGC 480
CTGACTATAT TTTTTTGTCC TAAATTTGCA AGCACACACC CACAAAGCTG CGGTCTTGAC 540
CGGTATTCTT TATAGAGCGC AATGGAGTGA GCTGAGTGTC TAAACGATTT CCCTAATTCA 600
TCTGATAGCA GAGGCGCTCT CCTAATTGGC GAAGAGCTGC CTCATGTCCG CAACTTTTTG 660
GCAGAGTGAA TTCCACAGCT TTGTGTGTGT GTGTGGGGGG GGGTGTAAGG GGTGTCTAAA 720
ACTTTCGGTC TCCTACTATT CTGTATCTCG ACCGGTTGGT TTTACACCCC GGCTCATCTC 780
ATCAACGCAA ACACCCCCAC TCTCCTATGG ACCCAAGGAC CTGACGTGGG GGAAGGTGGA 840
CATTAGGAAT GTCAGAAACC TAGAGTCCAC GCTCCTCCTC TCCATCTTTC CACGAGTTTG 900
GGAAACTTCT TGGCTGCGAA GACTTTGACC CACATCTGCA TTTCTCAGCC CCAGCTTCCA 960
AAAGTGCTGC AGGTTCGGGA GGGGAGACCT CAGTCCTCCT TTGTGAGGCT TGTTTGCGTT 1020
GAGGGATTGG CAGCGATGGC TTCCAGATGG GCTGAAACCC TGCCCGTATT TATTTAAACT 1080
GGTTCCTCGT GGAGAGCTGT GAATCGGGCT CTGTATGCGC TCGAGAAAAG CCCCATTCAT 1140
GAGAGGCAAG GCCCAGTGGG TCCCCCCGAC TCCCCGACCC CCCTCTCCCA CAATATATCC 1200

CCCCTCCCTG TGCCCGCCTG CCGCCACCTC CCGGGCTCCG GCCCCGCGCG CAGCGGCGAC 1260
GAAGCAACAC AGTTCCCCGA AAGAGGTAGC TTTTTAATTG GCCAGCCACA AAGAATCACT 1320
TATGCCGCAC GGCGGTAACG AGGGGAACCG GATCGGGCGG CCAGGATGCT ATCTGTGTAG 1380
CCCTTTTCGT GCCACAATTA GGGTGGTGCT GGCTTCCTCC GACCGCACCT AGGCGATCTG 1440
GTTACACTGT TGGCTCCTTT CTTGGGCAGT CATTTAATCC TACTTTTTAC TCTACGAATG 1500
TCTGTCTGAT GGAGGGCTGT GTCCGGAGCC CCATCCACAA AGAGTCAGCC AGCAGCTCTC 1560
ACACCCGGCT GGATCTCATA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT 1620
TAAGCCAGCC AAGCTAGCTT GCGCAAGCTA GCTTGCGATC CGTAAAAATG TGTGAGAGTT 1680
ACAAAATGTC TTCCGGGCTA AGATCCGACA GCCATGGTCC AAAGAAGACT TCGGCACTGC 1740
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AATAGGCTGG GAAGATGCAA GAATGGCATT TTACATAAAG AACACTCTCT CCTTTTCCAG 1860
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AAAGTTCCCT AGAGGACCAT CTCACTTCTA AAGAAAAGGT GTATTCGGGG ACCCATCCTC 2160
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GCGTTGCTCT CTCCAGGCTA AGGGCACCCA CTCCCCTGGA GATTCCTGAA CCTGGGCCAG 2520
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CCGATCAGGC CACTGGGCAC ATCGGGGGCG GGAAGCTGGG CTCACCAAAG GGGCGACTGG 3180
GCTTGGCAGG TGTGGGCTCT GGTCCGGCCT GGGCAGGCTC CGGGGGCGGG GTCTCAGGTT 3240
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GCCGGGTCTC CTGCCTCCTC CTCCTGCTCC TAGAGCCTCC TGCATGAGGG CGCGGTAGAG 3420
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GTCTCATCAT TTTGGCAAAG AATTCACTCC TCAGGTGCAG GCTGCCTATC AGAAGGTGGT 7620
GGCTGGTGTG GCCAATGCCC TGGCTCACAA ATACCACTGA GATC 7664

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGAGGGCCTG CTCGATCTCC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCATTCCAC CACTGCTCCC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGCACCCTT CTCATGACCT C 21

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGGTGTAG ATGGGCGCAT CG 22

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCGGGGTCTC AGGTTACAGC C 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCCTCTGGC CTGCTGGCTC ATG 23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAGGAGAGTC TTGCCTGTAT CCTC 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 1521 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
CAAGATGCAT CCAGGGGTCC TGGCTGCCTT CCTCTTCTTG AGCTGGACTC ATTGTCGGGC     60
CCTGCCCCTT CCCAGTGGTG GTGATGAAGA TGATTTGTCT GAGGAAGACC TCCAGTTTGC    120
AGAGCGCTAC CTGAGATCAT ACTACCATCC TACAAATCTC GCGGGAATCC TGAAGGAGAA    180
TGCAGCAAGC TCCATGACTG AGAGGCTCCG AGAAATGCAG TCTTTCTTCG GCTTAGAGGT    240
GACTGGCAAA CTTGACGATA ACACCTTAGA TGTCATGAAA AAGCCAAGAT GCGGGGTTGT    300
CGATGTGGG TGAATACAATG TTTTCCCTCG AACTCTTAAA TGGTCCAAAA TGAATTTAAC    360
CTACAGAATT GTGAATTACA CCCCTGATAT GACTCATTCT GAAGTCGAAA AGGCATTCAA    420
AAAAGCCTTC AAAGTTTGGT CCGATGTAAC TCCTCTGAAT TTTACCAGAC TTCACGATGG    480
CATTGCTGAC ATCATGATCT CTTTTGGAAT TAAGGAGCAT GGCGACTTCT ACCCATTTGA    540
TGGGCCCTCT GGCCTGCTGG CTCATGCTTT TCCTCCTGGG CCAAATTATG GAGGAGATGC    600
CCATTTTGAT GATGATGAAA CCTGGACAAG TAGTTCCAAA GGCTACAACT TGTTTCTTGT    660
TGCTGCGCAT GAGTTCGGCC ACTCCTTAGG TCTTGACCAC TCCAAGGACC CTGGAGCACT    720
CATGTTTCCT ATCTACACCT ACACCGGCAA AAGCCACTTT ATGCTTCCTG ATGACGATGT    780
ACAAGGGATC CAGTCTCTCT ATGGTCCAGG AGATGAAGAC CCCAACCCTA AACATCCAAA    840
AACGCCAGAC AAATGTGACC CTTCCTTATC CCTTGATGCC ATTACCAGTC TCCGAGGAGA    900
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TGTTCGCGTC ATGCCAGCAA ATTCCATTTT GTGGTGTTAA GTGTCTTTTT AAAAATTGTT   1440
ATTTAAATCC TGAAGAGCAT TTGGGGTAAT ACTTCCAGAA GTGCGGGGTA GGGGAAGAAG   1500
AGCTATCAGG AGAAAGCTTG G                                             1521
```

(2) INFORMATION FOR SEQ ID NO:19

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 7 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Pro Arg Cys Gly Xaa Pro Asp

1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
His Glu Xaa Gly His Xaa Xaa Xaa Xaa Xaa His Ser
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met His Pro Gly Val Leu Ala Ala Phe Leu Phe Leu Ser Trp Thr His
 1               5                  10                  15
Cys Arg Ala Leu Pro Leu Pro Ser Gly Gly Asp Glu Asp Asp Leu Ser
            20                  25                  30
Glu Glu Asp Leu Gln Phe Ala Glu Arg Tyr Leu Arg Ser Tyr Tyr His
        35                  40                  45
Pro Thr Asn Leu Ala Gly Ile Leu Lys Glu Asn Ala Ala Ser Ser Met
    50                  55                  60
Thr Glu Arg Leu Arg Glu Met Gln Ser Phe Phe Gly Leu Glu Val Thr
65                  70                  75                  80
Gly Lys Leu Asp Asp Asn Thr Leu Asp Val Met Lys Lys Pro Arg Cys
                85                  90                  95
Gly Gly Val Asp Val Gly Glu Tyr Asn Val Phe Pro Arg Thr Leu Lys
            100                 105                 110
Trp Ser Lys Met Asn Leu Thr Tyr Arg Ile Val Asn Tyr Thr Pro Asp
        115                 120                 125
Met Thr His Ser Glu Val Glu Lys Ala Phe Lys Lys Ala Phe Lys Val
    130                 135                 140
Trp Ser Asp Val Thr Pro Leu Asn Phe Thr Arg Leu His Asp Gly Ile
145                 150                 155                 160
Ala Asp Ile Met Ile Ser Phe Gly Ile Lys Glu His Gly Asp Phe Tyr
```

```
                165                 170                 175
Pro Phe Asp Gly Pro Ser Gly Leu Leu Ala His Ala Phe Pro Pro Gly
            180                 185                 190
Pro Asn Tyr Gly Gly Asp Ala His Phe Asp Asp Asp Glu Thr Trp Thr
        195                 200                 205
Ser Ser Ser Lys Gly Tyr Asn Leu Phe Leu Val Ala Ala His Glu Phe
    210                 215                 220
Gly His Ser Leu Gly Leu Asp His Ser Lys Asp Pro Gly Ala Leu Met
225                 230                 235                 240
Phe Pro Ile Tyr Thr Tyr Thr Gly Lys Ser His Phe Met Leu Pro Asp
                245                 250                 255
Asp Asp Val Gln Gly Ile Gln Ser Leu Tyr Gly Pro Gly Asp Glu Asp
            260                 265                 270
Pro Asn Pro Lys His Pro Lys Thr Pro Asp Lys Cys Asp Pro Ser Leu
        275                 280                 285
Ser Leu Asp Ala Ile Thr Ser Leu Arg Gly Glu Thr Met Ile Phe Lys
    290                 295                 300
Asp Arg Phe Phe Trp Arg Leu His Pro Gln Gln Val Asp Ala Glu Leu
305                 310                 315                 320
Phe Leu Thr Lys Ser Phe Trp Pro Glu Leu Pro Asn Arg Ile Asp Ala
                325                 330                 335
Ala Tyr Glu His Pro Ser His Asp Leu Ile Phe Ile Phe Arg Gly Arg
            340                 345                 350
Lys Phe Trp Ala Leu Asn Gly Tyr Asp Ile Leu Glu Gly Tyr Pro Lys
        355                 360                 365
Lys Ile Ser Glu Leu Gly Leu Pro Lys Glu Val Lys Lys Ile Ser Ala
    370                 375                 380
Ala Val His Phe Glu Asp Thr Gly Lys Thr Leu Leu Phe Ser Gly Asn
385                 390                 395                 400
Gln Val Trp Arg Tyr Asp Asp Thr Asn His Ile Met Asp Lys Asp Tyr
                405                 410                 415
Pro Arg Leu Ile Glu Glu Asp Phe Pro Gly Ile Gly Asp Lys Val Asp
            420                 425                 430
Ala Val Tyr Glu Lys Asn Gly Tyr Ile Tyr Phe Phe Asn Gly Pro Ile
        435                 440                 445
Gln Phe Glu Tyr Ser Ile Trp Ser Asn Arg Ile Val Arg Val Met Pro
    450                 455                 460
Ala Asn Ser Ile Leu Trp Cys
465                 470
```